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(54) Title: POLYNUCLEOTIDE ASSAY REAGENT AND METHOD

(57) Abstract

A diagnostic reagent and system for determination of a single-stranded polynucleotide analyte having a selected target sequence. The reagent includes a solid support and multiple support-bound polymers designed to bind specifically to the analyte. Each polymer is composed of a sequence of base-complementary recognition moieties which can bind specifically to corresponding in-sequence bases in the analyte target sequence, and an unbranched, stereoregular backbone whose charge density is substantially less than that of the polynucleotide analyte. A reporter in the system contains a polycationic tail adapted to bind to the analyte, under conditions in which the reporter does not bind to the less densely charged polymers, and reporter groups by which the presence of the reporter can be detected.

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POLYNUCLEOTIDE ASSAY REAGENT AND METHOD

1. Field of the Invention

The present invention relates to a
5 polynucleotide diagnostic system and method.

2. Background

Two general types of polynucleotide diagnostic
systems, both based on hybridization between
complementary segments of a polynucleotide probe and a
10 single-strand polynucleotide analyte, have been
developed. In the first type, the polynucleotide
analyte is made single stranded and fixed to a solid
support, such as a nitrocellulose filter. The support
is then reacted, under complementary-strand annealing
15 conditions, with a reporter-labeled probe which is
complementary to a target base-sequence region of the
analyte. After washing several times to remove unbound
probe, the solid support is analyzed for the presence of
reporter. This system has not been entirely
20 satisfactory, particularly in clinical situations, where
assay simplicity and sensitivity are required. The
procedure used in fixing single-strand polynucleotide
material to the solid support is somewhat involved and
time consuming. The sensitivity of the system is
25 limited because, in the usual case, each analyte
molecule hybridizes with a single probe molecule and
each probe generally contains roughly one hundred
reporter moieties.

A second type of polynucleotide diagnostic
30 system involves two analyte-specific probes which are
each complementary to a distinct region of the analyte
polynucleotide. The first probe is linked to a solid
support, and the second probe is free in solution and

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carries multiple reporter molecules. In practice, the
analyte is mixed with the two probes under conditions
which allow annealing of complementary polynucleotide
strands, including annealing of both the immobilized and
5   reporter-carrying probes to the polynucleotide analyte,
to attach the reporter to the solid support by means of
the analyte.

Although the dual-probe system avoids the
problem of having to fix the test nucleic acid material
10 to a solid support, nevertheless, the method has a
number of limitations. First, when the test nucleic
acid is derived from duplex nucleic acid, as is often
the case, hybridization between the analyte
polynucleotide and its complementary strand competes
15 with the hybridization between the analyte and the two
probes. Further, since the two-probe system relies on
higher order kinetics than is the case for single-probe
systems, it is inherently slower than a single-probe
system. Also, the need for two different probes
20 increases the cost of the system. Finally, in terms of
test sensitivity, the dual-probe system suffers the same
limitation as the single-probe system mentioned
above--each analyte polynucleotide binds only one
"reporter" probe.

25

3. Summary of the Invention

It is a general object of the invention to
provide, for use in detecting a polynucleotide, a
diagnostic system and method that substantially overcome
30 above-discussed problems and limitations associated with
the prior art.

It is another object of the invention to
provide, in such a system, a reagent having a
polynucleotide-binding polymer adapted to form a

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base-specific duplex structure with a single-stranded
polynucleotide analyte, under conditions in which
complementary polynucleotide strands remain in the
single-stranded state. The analyte binding can

5 therefore be performed under conditions in which
sequence-specific pairing between the reagent polymer
and analyte occurs without competition from
analyte-complementary strand annealing in the assay
reaction mixture.

10 It is another object of the invention to
provide in such a system, a reporter adapted to bind by
electrostatic forces to the backbone of an analyte
polynucleotide, under conditions in which reporter
binding to the reagent polymer does not occur. The

15 system therefore has the capacity for very high signal
levels by virtue of the relatively large number of
reporter molecules which can combine with each
polynucleotide analyte molecule.

Providing a polynucleotide diagnostic method
20 which is rapid, convenient, and sensitive is yet another
object of the invention.

The invention includes a diagnostic reagent for
use in detecting an analyte polynucleotide having a
defined target base sequence. The reagent comprises a

25 solid support, and linked to the support, multiple
polynucleotide-binding polymers. Each polymer is
composed of a sequence of base-complementary recognition
moieties, each of which is adapted to hydrogen bond to
corresponding, in-sequence base of the target region of

30 the analyte, under selected binding conditions, and an
unbranched, substantially stereoregular backbone (1)
supporting the recognition moieties at positions which
allow hydrogen bonding between the recognition moieties
and the corresponding in-sequence bases of the target,

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and (2) having a backbone charge density which is
substantially less than that of the analyte under the
selected binding conditions. In a prferred embodiment
of the invention, the recognition moieties include
5 purine and pyrimidine bases and the backbone is composed
of a series of backbone moeities joined by achiral,
predominantly uncharged linkages. In another embodiment
of the invention, the same recognition moieties are
carried on a backbone whose linkages include uncharged,
10 stereoisomerically defined or achiral linkages
alternating with negatively charged, achiral linkages.

A diagnostic system for detecting a
polynucleotide analyte having a defined target sequence
includes the diagnostic reagent and a reporter having a
15 polycationic tail adapted to bind by electrostatic
attraction to the charged backbone of the polynucleotide
analyte, but not to the substantially less charged, or
uncharged, backbone of the reagent polymers under
preselected binding conditions. One or more reporter
20 groups attached to the polycationic tail are adapted to
produce a signal by which the presence of the reporter
can be detected.

In the method of the invention, the
polynucleotide analyte is added to the diagnostic
25 reagent under conditions in which the analyte is in a
single-strand form and which allow sequence-specific
pairing between the analyte and the reagent polymers.
After the analyte/polymer annealing reaction, the
reagent is washed to remove unbound test material. The
30 reagent and bound analyte are then reacted with the
reporter, under preselected conditions in which the
polycationic tail in the reporter binds electro-
statically to the charged backbone of the polynucleotide
but not to the substantially less charged or uncharged

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backbone of the reagent polymers. The reagent is again washed to remove unbound reporter. The presence and/or amount of analyte bound to the reagent is determined in situ by measuring the reporter signal associated with

5 the reagent-bound analyte, or the reporter is eluted from the reagent/analyte and the reporter signal in the eluate is measured.

These and other objects and features of the present invention will become more fully apparent when

10 the following detailed description of the invention is read in conjunction with the accompanying figures.

Brief Description of the Figures

Figure 1 shows preferred purine and pyrimidine

15 structures used in forming polymer molecules of the invention;

Figure 2 shows preferred purine-like and pyrimidine-like recognition moieties used in forming the polymer molecules;

20 Figure 3 shows preferred cyclic backbone moities used in forming the polymer molecules;

Figure 4 shows preferred acyclic backbone moieties used in forming the polymer molecules;

Figures 5A and 5B illustrate two preferred

25 subunit assembly schemes for coupling N_1---N_2 type subunit backbones;

Figure 6 shows the backbone structures of subunit dimers A-A through D-D formed in accordance with the methods illustrated in Figures 5A and 5B, and two

30 cyclic backbone structures formed in accordance with the methods illustrated in Figures 7A and 7B;

Figures 7A-7C illustrate three preferred subunit assembly schemes for coupling N_1---E type subunit backbones; and

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Figure 8 shows preferred acyclic backbone structures of subunit dimers formed in accordance with the methods illustrated in Figures 7A-7C; and

Figure 9 shows the sythesis of a
5 tetranucleotide analog having methylphosponate backbone linkages alternating with a phosphotriester linkage;

Figure 10 illustrates reactions for attaching spacer-arm molecules to a solid support;

Figure 11 shows a general method for synthesis
10 of polyamines from N-methyl amino acids;

Figure 12 shows a method for synthesis of polyamines having a terminal primary amino group;

Figure 13 shows the preparation of the quaternary ammonium cationic tail having a terminal
15 primary amino group;

Figure 14 illustrates a reaction for preparing a multi-charge enzymatic reporter according to an embodiment of the invention;

Figure 15 illustrates a reaction for preparing
20 a two-charge fluorescent reporter according to an embodiment of the invention; and

Figure 16 illustrates various components involved in the diagnostic system and method of the invention.

25

Detailed Description of the Invention

The diagnostic system of the invention includes a solid support reagent composed of a solid support
30 carrying a plurality of nucleotide-binding polymer molecules. The polymeric composition of the invention is designed to bind, with a selected binding affinity, to a polynucleotide containing a target sequence of bases. The composition is composed of

non–homopolymeric, substantially stereoregular molecules
or species of the form:

$$
\begin{array}{cccc}
R_1 & R_2 & R_3 & R_n \\
| & | & | & | \\
B \sim B \sim B \sim \ldots B,
\end{array}
$$
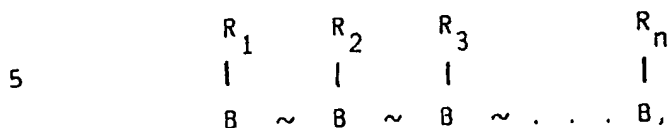
where $B-R_i$ are a series of base-specific subunits
containing a backbone moiety B and a recognition moiety
R_i which is selected to bind specifically by
Watson/Crick base pairing to a corresponding,
in-sequence base in the target sequence, and the
subunits are joined through their backbone moieties
predominantly achiral, substantially uncharged bonds.
The design and selection of suitable subunits for use in
constructing the polymer species will be be described in
Section I below. Methods for coupling subunits through
achiral linkages are described in Section III, with
subunit–protective group strategies involved in subunit
synthesis being discussed in Section II.

The polymer species are synthesized by
sequential subunit coupling, to form polymer molecules
of a selected length and sequence. As will be described
in Section IV, the length of the target sequence and
polymer sequence is selected to achieve a desired
binding specificity. The binding affinity of the
polymer for the target can be selectively varied by
several strategies discussed in Section IV, including
the choice of recognition moieties which are capable of
forming either three (for greater binding affinity) or
two (for lesser affinity) base-paired hydrogen bonds
with the corresponding target base. The resulting
composition contains polymer species or molecules, all
having substantially the same sequence of subunits and

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substantially the same sequence of intersubunit linkage
types. In functional terms, all of the polymer species
have substantially the same binding affinity for the
target polynucleotide. Methods for assembling the
5 polymer, once a desired subunit sequence is selected,
are given in Section V.

The polymer composition is useful in a novel
solid-phase diagnostic system which is described in
Section VI-VII. This system is based on the binding of
10 analyte polynucleotide molecules to support-bound
polymer molecules and subsequent elctrostatic attachment
of multiple polycationic reporter molecules to the
polynucleotide, to give an amplified reporter signal for
each bound analyte molecule.

15

I. Subunit Structure

A. The Recognition Moiety
The design of the subunits $B-R_i$ which are
20 suitable for use in forming the polymer species of the
invention involves a number of structural and/or
stereochemical criteria which must be met both by the
backbone and recognition moieties and by the linkage
between the two. The design requirement of the
25 recognition moiety will be considered first.

The recognition moiety of each subunit must
provide two or three hydrogen-bonding groups held in an
unambiguous configuration adapted for hydrogen bonding
to two or three of the Watson/Crick hydrogen bonding
30 sites on a specified in-sequence base of the target
genetic sequence. To avoid undesired mispairing between
recognition moiety and its corresponding target base,
under the conditions of use, (1) the tautomeric state of
the recognition moiety should be relatively stable, and

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(2) the recognition moiety should have a structure which
provides a relatively rigid arrangement of the hydrogen-
bonding groups. Such rigidity is best afforded by a
ring structure having the polar hydrogen-bonding groups
5   either forming part of the ring or directly attached to
the ring.

The preferred recognition moiety structures
include purine, purine-like, pyrimidine, and
pyrimidine-like structures which are designed to form
10   Watson/Crick base pairing, through either two or three
hydrogen bonds, with selected polynucleotide bases. The
group of subunits used in polymer synthesis includes at
least two recognition moieties which are base-specific
for different polynucleotide bases, and preferably one
15   or more recognition moieties for each of the four bases
in a natural DNA or RNA polynucleotide. Also, as will
be seen below, it is desirable to provide one group of
recognition moieties, R_i, which are capable of
base-pairing with nucleotide bases through two hydrogen
20   bonds, and a second group, R_j which are capable of
binding with the same bases through three hydrogen
bonds. Figure 1 shows exemplary purine and pyrimidine
type recognition moieties. The purine structures 1 and
2 are designed to bind to thymine or uracil bases,
25   structures 3-6, to guanine bases; structures 7-9, to
cytosine bases, and structures 10-12, to adenine bases.
Structures 1, 4, 5, 6, 8, and 10-12 are R_i type
moieties adapted to bind to corresponding in-sequence
bases through two hydrogen bonds, and the remaining
30   structures are R_j type moieties which form three
hydrogen bonds on base pairing. As will be seen below,
purine and pyrimidine nucleosides are useful in
synthesizing a variety of other subunits which are
suitable for use in polymer synthesis. These subunits,

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modified if necessary with amine protective groups, can
be obtained from commercial sources, or prepared
according to known methods, such as those described or
referenced in Example 1.

5   A number of purine-like or pyrimidine-like
structures in which the recognition moiety is joined to
the backbone through an annular carbon are shown in
Figure 2. These structures have base pairing
specificities and hydrogen bonding properties like those
10 of the analogous structures shown in Figure 1.
Although the binding properties of a polymer containing
a carbon-linked recognition moiety may not be
significantly different from that of a polymer whose
recognition moities are nitrogen-linked, as in Figure 1,
15 subunits incorporating the carbon-linked moieties
generally require a greater synthetic effort than do
subunits containing preformed purine and pyrimidines.
However, for some subunit syntheses, such as the
synthesis of the achiral acyclic backbone subunits
20 described in Example 10, the carbon-linked recognition
moieties provide a useful starting material.

B.   The Backbone Moiety
          The backbone moiety of each subunit has the
25 general form N_1---E, or N_1---N_2, where N_1 and
N_2 are nucleophilic groups and E is an electrophilic
group. Based on ease of subunit coupling and required
stability of the resultant intersubunit linkage, as
discussed below, the preferred nucleophilic groups are
30 amino, hydroxyl and hydrazino; and the preferred
electrophilic groups, and/or electrophilic linking
agents, are derivatives of carbonic, thiocarbonic,
carboxylic, and sulfonic acids.

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Backbone moieties can have either a cyclic or an acyclic structure. While the total number of possible backbone moiety structures is very large, nevertheless, only a rather limited number of structures

5   are of actual practical value due to a number of factors. The initial procedure by which promising backbone moieties were selected was as follows.

As a first conditions, only those cyclic backbone moieties were considered which consisted of, or

10   could be readily derived from, deoxyribose or ribose. This limitation is a practical one and reflects the difficulty and corresponding greater expense of de novo synthesizing ring structures having multiple chiral centers. In general prospective backbone moieties and

15   intersubunit linkages expected to be suitable for polymers were selected on the basis of one or more of the following factors: feasibility of synthesis based on known reactions; expected ease of synthesis from available starting materials; simplicity of structure;

20   and expected stability of the final backbone.

Initial screening of promising backbone moieties and intersubunit linkages was performed as follows. Space-filling CPK molecular models of duplex DNA and RNA were constructed according to parameters

25   determined by x-ray diffraction of oligodeoxyribo nucleotides in the B-form and oligonucleotides in the A-form. In each of these constructed duplexes one of the two sugar-phosphate backbones was removed. Next, each prospective backbone was attached, if possible, to

30   the sites on the bases form which the original sugar-phosphate backbone had been removed. Each resulting polynucleotide/polymer duplex was then examined for coplanarity of the Watson/Crick base-pairs, torsional and angle strain in the prospective polymer

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backbone, degree of distortion imposed on the nucleic
acid strand, and interstrand and intrastrand nonbonded
interactions. Special attention was paid to whether or
not each amide-containing backbone could readily adopt a
5   conformation wherein its amide moieties were planar.
This is important because of the substantial energy cost
required to force an amide into a nonplanar conformation.
        These initial studies verified that the
required unit backbone length (i.e., the N_1---E
10 spacing in an N_1---E or activated N_1---N_2-E
subunit) is 5-7 atoms for backbone moieties constituting
or derived from deoxyribose or ribose (cyclic backbone
structures), with a 6-atom length being optimal.
        Subunit structure judged acceptable in the
15 above modeling studies were then assessed for
feasibility of synthesis (on the basis of key synthetic
reactions reported in the literature, or reactions
carried out on model compounds, and/or via actual
synthesis of the subunits) and stability of the
20 assembled polymer backbone (preliminary stability
studies were generally carried out with suitably linked
model compounds or subunit dimers). These types of
studies were used to further restrict the number of
candidate backbone structures. From this restricted
25 candidate pool the cyclic backbone structures A-G shown
in Figure 3 were ultimately selected as preferred
structures.
        In this figure, subunits A-D containing
N_1----N_2 type cyclic backbone moieties include:
30 2'-deoxyribonucleosides (structure A);
2'-deoxyribonucleosides substituted at the 5' and 3'
positions with an amino group (structures B and C,
respectively); and morpholino derivatives of
ribonucleosides (structure D). Subunits containing

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N_1---E-type backbone moieties include
2'-deoxyribonucleosides substituted at their 5'
positions with one and two-carbon acids (structures E
and F, respectively); and N-aminomorpholino derivatives
5 of ribonucleosides substituted at their 5' positions
with carboxylic acid (structure G) or sulfonic acid
(structure H).

The same molecular modeling approach applied to
acyclic (unbranched chain) backbone moieties showed that
10 unit backbone lengths ranging from 4-6 atoms are
acceptable, in terms of polymer conformation in
base-pair hydrogen bonding to single-stranded
polynucleotide, with a 5-atom backbone length being
optimal. This is in contrast to cyclic backbones, where
15 a 6-atom unit backbone length is optimal. Further, the
modeling work showed that the annular recognition moiety
cannot be directly attached to the linear backbone
moiety without severely inhibiting of coplanarity of the
complementary bases and introducing undesirable
20 interactions between the recognition moiety and the
backbone the minimum binding strain occurred when the
recognition moiety was linked to the backbone moiety by
a one-atom spacer. Two-atom, but not three-atom,
spacers may be tolerated, although increased degrees of
25 freedom between the recognition moieties and the linear
backbone may lead to mispairing with target bases with
two-atom spacers.

The acyclic backbone moiety structures I-N shown
in Figure 4, all of which contain a one-atom methylene
30 spacer between the backbone and recognition moieties,
gave a good modeling prediction for favorable
base-pairing to a complementray polynucleotide.
Analogous structures containing a two-carbon spacer
showed acceptable, but less favorable, binding

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conformation. The structures shown in the figure, and
the analogous two-carbon spacer structures, are
preferred acylcic backbone moieties because of their
general ease of synthesis. It should be mentioned,
however, that a number of other backbone moieties are
also entirely feasible and suitable -- although
generally not as easy and/or inexpensive to synthesize.

C. Backbone/Recognition Moiety Linkage

        As indicated above, the linkage or spacer
connecting the backbone and recognition moieties in the
polymer subunit must meet certain design criteria which
are effective to position the recognition moieties for
base-pair binding to the polynucleotide bases. In the
case of the cyclic backbone moieties shown in Figure 3,
modeling studies indicate that the most favorable
binding conformation in the polymer occurs when the
recognition moiety is attached directly to the 1' carbon
of the ribose or ribose-like structures, and to the
analogous 1' position of the morpholino structures.
That is, the moiety is attached at normal point of
attachment and with the normal stereoisomeric
configuration of purine or pyrimidine bases to the
ribose or deoxyribose groups of nucleosides. For the
acyclic structures, one-and two-carbon atom spacers are
required for placing the recognition moieties at
favorable binding positions, with one-atom spacers being
preferred, as discussed above.

        In order to achieve a stereoregular polymer
configuration required for uniform binding affinity of
the polymer molecules to a polynucleotide target, it is
also necessary that all of the backbone/recognition
moiety linkages be either of define chirality or
achiral. The linkages are considered to be of defined

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chirality, for purposes of definition herein, if each of
the linkages in any given subunit position in all of the
polymer molecules has the same stereoisomeric
configuration, or chirality. That is, although the
5   subunits in different sequence positions may have
different internal stereoisomer configurations
(including achirality at specific sequence positions),
all of the linkages at a given sequence position among
the polymer molecules have the same stereoisomeric
10   configuration. Usually defined chirality is most easily
achieved by using the same stereoisomeric configuration
in all of the subunits.

For the cyclic backbone moieties, the most
favorable binding occurs in nucleoside analogs having ↳
15   the natural D-steriosomeric configuration shown in
Figure 3. Subunits of this type are also readily
synthesized, as will be discussed below, using natural
D-nucleoside starting material. With reference to
Figure 4, it is seen that only structures I and L have
20   chiral linkages between the backbone and recognition
moieties. These subunits are readily synthesized in
homochiral form by using homochiral starting material ,
as will be described. For all of the other acyclic
structures shown in Figure 4, the one-atom linkages are
25   attached to backbone nitrogen atoms, are are therfore
achiral. Also, of course, the methylene spacers are
themselves achiral.

II. Protective Group Strategies
30   Because of the rather high reactivity of the
compounds used for activating and/or coupling the
subunits, it is generally desirable, and often
necessary, to protect the exocyclic ring nitrogens of
the recognition moieties. Selection of these protective

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groups is primarily determined by the type of
intersubunit linkage to be used in the polymer assembled
from these subunits, and secondarily determined by the
relative reactivity of the nitrogen to be protected.

5   Base-protected nucleosides are also useful
starting reagents in a number of the subunit synthesis
reactions to be described below. Methods for base
protecting a number of the more common ribo- and
deoxynucleosides from Example 1 are illustrated in

10   Example 2. The methods detailed in the example are
generally applicable for forming nucleosides with
amine-protective groups.

        When the intersubunit linkage to be used is
relatively stable to nucleophiles, and particularly to

15   ammonium hydroxide, then the standard base-protective
groups used for nucleic acid chemistry are suitable.
Such nucleophile-insensitive intersubunit linkages
include carbamate, amide, and sulfonamide linkages. The
corresponding nucleophile-sensitive protective groups

20   for the recognition moieties include: benzoyl for the N4
of C; benzoyl or p-nitrobenzoyl for the N6 of A; acetyl
or isobutyryl for the N2 of G; and N2,N6-bisisobutyryl
for 2,6-diaminopurine residues. Removal of these groups
after completion of polymer assembly is effected by

25   treatment with ammonium hydroxide.

        In contrast, when the intersubunit linkage to
be used is sensitive to nucleophiles, such as ammonium
hydroxide, suitable protective groups are those which
can be removed by strong non-nucleophilic bases — via a

30   β elimination mechanism. Such nucleophile-sensitive
intersubunit linkages include: carbonate; ester; and,
to a lesser extent, triocarbamate linkages. Suitable
protective groups for the recognition moieties removable
via β elimination include: 2-(4-nitrophenyl)ethoxy

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carbonyl or 2-(phenyl sulfonyl)ethoxycarbonyl for both
the N4 of C and the N6 of A; and the 9-fluorenyl
methoxycarbonyl for the N2 of G and the N2 and N6 of
2,6-diaminopurine residues. Removal of these groups

5 after completion of polymer assembly is effected by
treatment with the strong nonucleophilic base
1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), under
stringently anhydrous conditions.

In regard to temporary protection of a
10 nucleophile of the backbone moiety (generally N1 in the
above structures), in the general polymer assembly
strategy employs selected backbone-protective groups
which are readily cleared by mild acid. One primary
criterion for selection of protective groups is that
15 they be adequately stable, but not so stable that
conditions required for their removal would damage the
growing polymer. A principal problem in the case of
polymers assembled from cyclic backbone moieties is the
particular acid sensitivity of the glycosidic bond
20 linking protected purine residues to the C1 of their
backbone moieties. A secondary criterion for selection
of the backbone protective group is that it be easily
introduced. Based on the above, the following backbone-
protecting groups are preferred: for primary
25 hydroxyls, the di(p-methoxy)trityl group; for primary
amines, p-methoxytrityl; and for a secondary amine (as
in morphilino-type backbone moieties), the
phenylisopropoxycarbonyl group. These protective groups
can be readily removed by treatment with 0.2 M
30 dichloroacetic acid in dichloromethane.

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III. Subunit Synthesis

A. Cyclic Backbone Moieties

Subunits having a deoxydeoxynucleoside subunit
5   structure (structure A in Figure 3) can be obtained from
commercial sources or prepared via literature methods,
as described in Example I. The subunits include the
following ribosides and deoxyribosides, which are
identified according to the struture numbers of the
10   recognition moieties given in Figure 1: adenosine and
deoxyadenosine (structure 1); 2,6-diaminopurine riboside
and deoxyriboside (structure 2); cytodine and
deoxycytodine (structure 3); 4-methoxy-2-pyrimidinone
deoxyriboside (structure 4); 2-hydroxy-5-methyl
15   pyrimidine deoxiriboside (structure 5);
2-hydroxypyrimidine riboside (structure 6); guanosine
and deoxyguanosine (structure 7); inosine and
deoxyinosine (structure 8); thioguanosine and
deoxythioguanosine (structure 9); uradine and
20   deoxyuridine (structure 10); thymidine and
5-methyluridine (structure 11;) and 5-halouridines and
5-halodeoxyuridines (structure 12).

The 5'-amino-2',5'-dideoxyribonucleosides
(structure B in Figure 3) are prepared according to
25   methods detailed in Example 3. Briefly, the selected
deoxyribonucleosides, base-protected if necessary, are
reacted with triphenyl phosphine, carbon tetrabromide,
and lithium-azide to form the corresponding
5'-azidonucleoside, which is then reduced by
30   hydrogenation in the presence of a palladium on carbon
catylyst. The nucleosides may be obtained as in Example
1, and base-protected as in Example 2. The
stereochemistry of the reactant nucleosides is preserved
in forming the 5' amino nucleoside analogs.

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An alternative reduction method is used in
reducing the azide group of 5'-azido-5-bromo uridine, as
described in Example 3.2. Here non-catalytic
hydrogenation is needed to prevent removal of the ring
5 bromine atom. In forming the 5'-amine guanosine
compound, the azide is placed on the 5' position by
first tosylating the 5' hydroxyl group, then displacing
with azide, as detailed in Example 3.2.

The 3'-amino-2',3'-dideoxyribosnucleosides
10 (structure C in Figure 3) are prepared according to
methods detailed in Example 4. Briefly, thymidine which
is protected at its 5' hydroxyl is tosylated at its 3'
hydroxyl, and this is followed by an intramolecular
displacement involving the 2 oxy base substituent. The
15 resulting ring is now opened by treatment with azide,
yielding a 3' azido analog of the correct stereoisomeric
form. This analog can be be converted to a
selected-base analog by reacting the azide compound
with a selected purine or pyrimidine base. The latter
20 may be base protected, if necessary, for subsequent
subunit coupling reactions, as described below. The
thymidine or other azide analog is then reduced to
produce the desired 3' amine nucleoside. The
stereochemistry is of the thymidine starting material is
25 preserved in the synthesis.

Synthesis of the morpholino-type subunit
derivatives represented by structure D in Figure 3 are
detailed in Example 5 for a variety of different
recognition moieties. Briefly, a selected nucleoside,
30 base-protected if necessary, is dissolved in an ammonium
salt, such as ammonium biborate, and then reacted with
sodium periodate to form transient 2', 3' dialdehydes,
which then close upon an ammonium ion to form morpholino
ring having 2' and 4' hydroxyls. The compound is then

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treated with sodium cyanoborohydride to remove the ring
hydroxyls. The ring nitrogen is preferably protected as
a 2-phenylisopropylcarbamate for subsequent subunit
coupling. The sterochemistry of the nucleoside starting
5 material is retained.

        Deoxyribose subunit structures having a 5'
acetic acid group (structure F in Figure 3) can be
prepared according the general synthetic scheme
described in Example 6, which details the synthesis of
10 the 5' acetic acid compounds represented by structure
F. Here a selected deoxyribonucleoside which is
protected at the 3' hydroxyl is converted to the 5'
aldehyde, and subsequently treated with a 2-carbon
Wittig reagent to give the unsaturated acetic acid
15 derivative. Reduction of the side-chain olefin gives
the desired 5' acetic acid compound. The reaction
preserves the sterochemistry of the starting nucleoside
material

        The analogous 5' formate compound (structure E
20 in Figure 3) is formed by treating the above 5'
nucleoside aldehyde with a one-carbon Wittig reagent,
and hydroslysing the resulting enol to form form the
corresponding formaldehyde derivative, which is oxidized
to the desired acid. The reaction preserves the
25 stereochemistry of the starting nucleoside material.

- -

B. Subunit Synthesis-Acyclic Backbones
        The 5-atom chain amino acid subunit represented
by Structure I in Figure 4 is prepared according to the
30 general procedures outlined in Examples 7-9. Briefly,
an (S)-5 carboxy pyrrolidone was converted to the
corresponding stereochemically pure
(S)-5-tosylmethyl-2-pyrrolidone by known methods, and
then reacted with a selected purine or pyrimidine, to

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form the corresponding (S)-5-methylpurine or pyrimidine pyrrolidone. The displacement reaction retains the original stereospecificity, so that the subunits which are formed are homoisomeric at the site of attachment of
5   the recognition moiety to the backbone carbon.

The purine used in subunit synthesis is preferably contains an electron-withdrawing group at the 6 position, to reduce the reactivity of the 1 and 3 ring nitrogens, i.e., to minimize pyrrolidone coupling at a
10   ring nitrogen. The pyrrolidone-derivized purine or pyrimidine may then be converted to the amine derivative, and base protected, if necessary, prior to the ring opening step which will be described below. Example 7.1 and 7.2 detail methods for forming the
15   cytosine pyrrolidone and its base-protected derivative. The adenosine pyrrolidone formed in Examples 7.3-7.5 employs 6-chloropurine as the starting material, and the corresponding pyrrolidone is converted to the adenosine derivative through azide formation as
20   described. The adenosine is base-protected, as in Example 7.6, prior to ring opening. A similar approach is used to generate base protected guanine pyrrolidone, starting from 2-amino-6 chloropurine, as described in Examples 7.7-7.9. Similar methods are used in
25   synthesizing 2,6-diaminopurine pyrrolidone, inosine pyrrolidone, and 2-hydroxypyrimidine pyrrolidone, also as detailed in Example 7.

The pyrrolidone is then treated via a series of reactions which cleave the pyrrolidone ring to form an
30   amino acid with a t-BOC protected amine. Examples 8.1-8.4 describe the synthesis of such t-BOC amino acids having one of a number of selected recognition moieties. In a final step (Example 5), the t-BOC protective group may be removed to form the

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corresponding amino acid represented by structure I in Figure 4. The amino acid may be used directly for subunit coupling, according to reactions given below. Alternatively, the t-BOC protected compound may be
5 activated at its acid group for use in subunit coupling reactions.

The strategy for forming the subunit represented by structure J in Figure 4 is outlined in Examples 10.1 and 10.2, for forming the cytodine and
10 uridine analog subunits, respectively. Briefly, in forming a pyrimidine type subunit, a 6-member heterocycle, such as a pyrimidine or nicotinate, is modified to contain a reactive methylene on the carbon ring position at which attachment to the backbone is
15 desired. As detailed in Example 10.1, the cytosine analog is formed by reacting 5-amino-5-bromopyrimidine to form the corresponding 5-position carboxaldehyde. In Example 12, the uridine analog is formed by converting 6-hydroxynicotinic esters to the corresponding benzylic
20 alcohols. This compound is converted to the aldehyde by reaction in the presence of manganese dioxide.

The aldehyde compound is then reacted with the desired amino acids, such as 4-aminobutyric acid, with reaction at the amine group producing an unstable imine
25 which is reduced to form the stable amine subunit. The secondary amine can be protected, for subunit coupling involving activation of the acid group, by a tBOC group as described in Example 10.1. The base attachment to the backbone at a backbone nitrogen, through a methylene
30 spacer, insures that the subunits lack chiral centers and so cannot be stereoregular.

To form the subunit structures shown at K in Figure 4, the C-carbon backbone analog of the above compound is prepared, using 3-aminoproprionic acid, and

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this compound is further treated with nitrous oxide to form the corresponding N-nitroso compound, which is subsequently reduced with H_2IPd in the presence of iron salts to give the hydrazino acid subunit.

5   III. Backbone Coupling Reactions

The coupling reactions used in forming the polymer molecules of the invention are stepwise reactions which join one selected subunit or subunit
10   sequence to another selected subunit or subunit sequence. For purposes of the present discussion, the coupling reactions will be described generally with respect to coupling a single subunit $B-R_1$ having a selected recognition moiety R_1 to another single
15   subunit $B-R_2$ having the same or a different selected recognition moiety R_2, to form a dimer of the form

$$\begin{array}{cc} R_1 & R_2 \\ | & | \\ B & B \end{array}$$
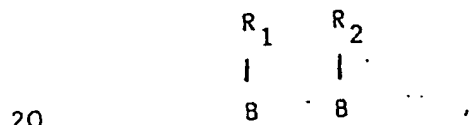
20

where R_1 and R_2 have the specific sequence shown.

A.   Subunit Coupling: N_1----N_2 Backbone Configurations

25   General methods for coupling subunits having an $N_1--------N_2$ backbone configuration are illustrated in Figures 5A and 5B. As will be recalled from Section I above, N_1 and N_2 are nucleophilic backbone groups, such as hydroxyl and amine groups, which can be
30   activated with an electrophile E, to form an activated N_1-E or N_2-E backbone group which can then react with a second N_1----N_2 type backbone moiety to form an $N_1----N_2-E-N_1----N_2$ backbone-linked dimer. The subunit backbones of this types which have been

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described specifically above are the cyclic backbone
structures A-D in Figure 3. In structures A and B, the
activated N_2 nucleophile is the 3' hydroxyl group in
structure C is the 5' hydroxyl and in structure D, the
6' position hydroxyl corresponding to the 5' position
hydroxyl in structure C.

    In a preferred coupling method, which is
illustrated in Figure 5A, a subunit having a selected
recognition moiety R_1 is activated with an
electrophile E. The star at the recognition moiety
indicates any required base protection. As seen in the
figure, the selected subunit is protected at its N_1
nucleophile, to ensure that (a) only the N_2
nucleophile is activated and (b) the activated subunit
cannot self-polymerize. In structures A, C, and D in
Figure 3, in which the backbone protection group is on
the 5' hydroxyl, the protective group is preferably an
acid-labile group, such as dimethoxytrityl (DMTO).

    The activating reagent shown in Figure 5A has
the general form:

$$Y-\overset{\overset{\textstyle X}{\|}}{C}-Y, \text{ where}$$
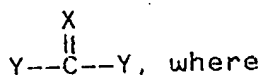
X is oxygen or sulfur, and C is an active electrophile
capable of reacting with a nucleophile, such as a
hydroxyl oxygen or amine nitrogen, with displacement of
Y, to form the activated subunit:

    Activating agents, such as bis-p-nitrophenyl
carbonate, which give the carbonyl activated subunit
(X=O), are used in forming carbonate and carbamate
subunit linkages. Similarly, activating agent, such as
thiocarbonyl-di-(1,2,4-triazole) (X=S) are used in
forming thiocarbamate linkages.

    Subunit activation reactions involving carbonyl
activated subunits are detailed in Example 11 for the

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.5'-protected, 2' deoxynucleosides represented at A in
Figure 4; in Example 12 for the 5'-protected amino
nucleosides represented at B in Figure 4; and in Example
13 for the N-protected morpholino-type subunits shown at

5 D in Figure 4. The general reaction conditions used to
activate the hydroxyl groups in these structures are
generally applicable; subunit activation reactions
involving thiocarbonyl-activated subunits are detailed
in Example 14 for the 5'-amino nucleosides represented

10 at B; and in Example 15, for the morpholino-type
subunits shown at D in Figure 3.

Following the activation reaction, the
activated complex is purified by conventional methods,
such as silica gel chromoatography, and then reacted

15 with a second subunit whose selected recognition moiety
R_2 will form the next in-sequence recognition moiety
in the completed.polymer. The coupling reaction is
preferably carried out under mild conditions in which
the activated group can react with backbone N_2 amine

20 groups, but not N_1 hydroxyl groups. Therefore, the
method is suitable for coupling subunits of the type
represented by structures B-D in Figure 4, but not
subunit structure A. An advantage of this coupling
method is that the second subunit -- which contains an

25 amine N_1 nucleophile and a hydroxyl N_2 nucleophile
-- will be coupled to the first activated subunit only
through the N_1 amine, so it is not necessary to
protect the N_2 backbone group. The resulting dimer
subunits are therefore coupled through an N_2-E-N_1

30 bond, as indicated in the figure.

The oligomer can be extended by repeating the
above steps of (a) activating the free N_2 nucleophile
in the second subunit, separating the activated species
from the activating agent, and coupling the activated

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compound with the next-in-sequence subunit, whose
backbone is unprotected. This method is used
particularly in forming short oligomer blocks by
solution methods which will be described below and which
5 are suitable for solid-phase block assembly.

For forming a polymer by solid-phase sequential
subunit addition, the second coupling method outlined in
Figure 5B is much preferred. The second method differs
from the first method in that polymer growth occurs by
10 addition of an excess of activated subunit to an
existing subunit or polymer chain, rather than, as in
the first method, by addition of an unactivated subunit
to an activated chain. In Figure 5B, the existing
subunit or subunit chain is shown by a subunit whose
15 recognition moiety is R_1 (second line in Figure 5B).
This subunit has a free N_1 backbone nucleophile and an
N_2 nucleophile which is protected by a preferably
acid-stable linkage to a solid support or by virtue of
being linked to a chain of subunits which are linked to
20 a solid support. Methods of forming cyclic backbone
subunits which are N_2 protected in this manner are
described generally in Examples 12-15. The first
subunit (the most recently added subunit in a growing
polymer) is now reacted with an activated subunit which
25 thereby becomes the next-in-sequence subunit in the
polymer. This activated subunit, which is activated at
its N_2 backbone site, and protected at its N_1 site,
preferably by an acid-labile protective group, is
prepared by methods described above with reference to
30 Figure 6.

As seen in Figure 5B, the coupling reaction
adds the N_2-activated second subunit to the
N_1-protected first subunit to couple the two through
an N_2-E-N_1 bond, and form a compound which is

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protected at both free backbone nucleophile sites. This
compound is now treated, for example by reaction with
acid, to deprotect the acid-labile N_1 protective group
on last-added subunit, and the procedure is repeated to
5 build up a desired sequence polymer.

It can be appreciated from the above that the
N_2-activated subunit which will form the
last-in-sequence subunit must be protected at its N_1
backbone site, to allow selective activation at the N_2
10 site and to prevent self-polymerization of the
activated compound. Also the N_1-deprotected subunit
which is to couple to the activated subunit should be
protected at its N_2 site to allow selective reaction
of its N_1 moiety with the N_2-activated subunit.
15 Therfore, since the reacting subunits are both protected
at one backbone site, the method is suitable for oupling
nucleosides (structure A in Figure 4) as well as
subunits whose cyclic backbones contain both amine and
hydroxyl backbone nucleophiles, such as structures B-D
20 in this figure. The reaction methods used in coupling
structure A subunits through a carbonate bond are
detailed in Example 12. Briefly, a subunit containing a
5' protected backbone moiety is activated at its 3'
hydroxyl, and reacted with another subunit (or growing
25 chain) which is protected at its 3' hydroxyl. The
coupling reaction is carried out in the presence of a
catalyst, such as N-methylimidazole or
N,N-dimethylaminopyridine, which is necessary for
forming the carbonate bond. For coupling subunits
30 containing structure B-D cyclic backbones, where
intersubunit carbamate or thiocarbamate bonds are
formed, much milder uncatalyzed coupling conditions,
such as those described with reference to the first
method above, are suitable.

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The advantage of this second coupling method,
for forming a polymer by solid-phase subunit addition,
is that a substantial molar exess of the activated
subunit can be added to the growing support-bound
5 polymer at each polymer-addition step, to achieve
subunit coupling to a very high percentage of the
support-bound polymers. This insures that a large
percentage of the support-bound polymers will contain
the complete sequence of subunits desired. By contrast
10 in the first method, where the growing polymer is
activated at its last-added subunit, the efficiency of
subunit addition is limited by the efficiency of the
activations steps.

Figure 6 shows the dimeric structures produced
15 by coupling the cyclic backbone subunits indicated at
A-A through D-D in Figure 4. The nucleoside subunits in
structure A in the figure are joined by a carbonate
(Y=O). In the remaining three structures B-D, the
subunits are joined by carbamate (Y=O) or thiocarbamate
20 (Y=S) bonds. As seen from the figure, all of the
subunit linkages are uncharged and achiral, i.e., do not
contain chiral centers. In addition, the linkages are
stable in aqueous medium, as judged by the ability of
polymers to resist hydrolysis over an extended period in
25 neutral aqueous solution.

According to another important feature of the
invention, the structures, when joined to form polymers
show acceptible Watson/Crick base pairing with
complementary polynucleotides.

30   Finally, according to another important feature
of the invention, polymers formed from the subunits are
stereoregular. This is achieved in the structures shown
by (a) using natural nucleosides or nucleoside
derivatives or synthetic nucleosides having the natural

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stereoisomeric configuration as subunits, and (b) joining the subunits by achiral intersubunit linkages. Exemplary coupling methods are detailed in Examples 11-15.

5

B.   Subunit Coupling: N_1----E Backbone Configurations

General methods for coupling subunits having an N_1-----E backbone configuration are illustrated in Figures 7A, 7B, and 7C. As will be recalled from

10   Section I above, N_1 is a nucleophilic backbone group, such as a hydroxyl or amine group, and E is an electrophile, such as a carboxyl or sulfonyl group, which, when activated, can react with a second N_1-----E type backbone to form an N_1-----E-N_1

15   -----E backbone-linked dimer. The subunit backbones of this types which have been described specifically above are the cyclic backbone structures E-H in Figure 3, and the acyclic backbone structures I-N in Figure 4.· In the cyclic structures E and F, the N_1 nucleophile is the

20   3' hydroxyl group, and in structures G and H, the amine attached to the 3' nitrogen on the morpholino ring. The E group in all of the cyclic structures is the carboxyl or corresponding sulfonyl group attached to the 5' position of the ribose ring, or to the 6' position of

25   the morpholino ring. In all of the structures shown in Figure 4, the N_1 and E groups are respectively, the backbone amine or hydrazine and carboxylic or sulfonic acid groups on the backbone moiety ends

The first coupling method, which is illustrated

30   in Figure 7A, is analogous to the method described above with reference to Figure 5A, where a first N_1-protected subunit is activated, then coupled directly to a backbone-unprotected subunit which forms the next-in-sequence subunit in the growing polymer.

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The P_1 protective group on the subunit N_1 is
preferably an acid-labile protective group, such as
t-butoxycarbonyl or a clevable linker bound to a solid
support. Methods for N_1-protecting cyclic backbone
5   structures are analogous to procedures described above
for cyclic structures A-D. Similar methods are
effective to protect the amine nitrogens in the acyclic
bonebone structures. Alternatively, if the polymer is
to be constructed on a solid phase support, as described
10 below, the backbone N_1 site may be protected by its
attachment to a solid support, with subunit addition to
the growing polymer occuring via the activated E
electrophile of the last-added subunit.

      The activation reaction is designed to yield an
15 activated or chain-terminal moiety of the form
N_1------E-X, where X is as described above with
reference to Figure 6, and the activated subunit has
much the same reactivity toward backbone nucleophilic
groups as does the activated subunit in the previously
20 described method. That is, the activation is designed
to produce much the same active coupling group as in the
N_1----N_2 type backbones, but where the reactive
carbonyl or sulfonyl group is provided by the backbone
itself rather than by a carbonyl or sulfonyl activating
25 reagent, as in the methods shown in Figure 6.

      The activation can be performed readily by
reacting the N_1----E type subunit with a carbodiimide
in the presence of p-nitrophenol, where the backbone
electrophile is a carbonyl group, or by reacting with a
30 carbodiimide in the presence of imidazole, 1,2,4
triazole or tetrazole, where the backbone is a
sulfonyl. Subunit activation reactions involving
carbonyl electrophilic groups are detailed in Examples
16 and 17. The general reaction conditions used to

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activate the N-amino-morphilino and linear chain
backbone carbonyl groups are generally applicable to the
other N_1----E type backbone structures shown in
Figures 3 and 4.

5   Following the activation reaction, the
activated complex is purified by conventional methods,
such as silica chromatography, or, in the case of a
support-bound activated chain, simply washed, and then
reacted with a second subunit whose selected recognition
10   moiety R_2 will form the next in-sequence recognition
moiety in the .completed polymer. _The coupling reaction
yields a dimer whose subunits are therefore coupled
through an $E-N_1$ bond, as indicated in the figure. As
above, both subunits must be suitably base protected
15   during the activation and coupling reactions.

          The polymer can be extended by repeating the
above steps of (a) activating the free E electrophile
in the· second .subunit, (b) separating the activated
species from the activating agent, and (c) coupling the
20   activated compound with the next-in-sequence subunit,
whose backbone is unprotected.

          The second coupling method, which is
illustrated in Figure 7B, is directly analogous to the
method described above with respect to Figure 5B. Here
25   a first subunit having an E-protected backbone is
reacted with a second subunit with an activated E group
and a protected N_1 nucleophile, to form a dimer linked
through an $E-N_1$ bond and protected at both free
backbone end groups. Where the polymer is being formed
30   by solid-phase synthesis, the P_2 protective "group"
takes the form of an acid-stable linkeage to a solid
support. The N_1-protected subunit is prepared and
activated as above. Coupling conditions generally

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follow those used in the above cyclic subunit coupling
reactions.

In the third coupling method, shown at Figure
7C, the n_1-protected and E-unprotected subunits (or
polymer units) are reacted together in the presence of a
suitable E-activating agent, such as a carbodiimide, as
indicated

Figure 8 shows the dimeric structures produced
by coupling the cyclic amd acyclic backbone subunits
indicated at E-K in Figures 3 and 4, and indicated at
E-E through K-K, repsectively. The nucleosides subunits
in structure F-F in Figure 3 are joined by an ester
linkage. In remaining cyclic backbone structure, G-G,
the subunits are joined through a hydrazid or sulfonyl
hydrazide linkage. All of the acyclic backbone
subunits, shown at I-I, J-J and K-K, are linked through
amide (E=carbonyl) or sulfonamide (E=sulfonyl) bonds.
As in the above-discussed cyclic backbone subunit
structures, all of the subunit linkages are uncharged
and achiral, and all of the bonds are reasonably stable
in aqueous medium, as judged by the known stability of
ester, hydrazide and amide linkages in solution.

According to another important feature of the
invention, the structures, when joined to form polymers
show Watson/Crick base pairing with complementary
polynucleotides.

Finally, as with the N_1-----N_2 backbone
subunits described above, all of the N_1-----E backbone
structures shown are stereoregular. This is due to (a)
the homochiral (structures E-H in Figure 3 and I and L
in Figure 4) or achiral (structures J, K, M, and N in
Figure 4) nature of the recognition moiety attachment to
the backbone moiety, and (b) the achiral subunit
linkage.

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C. Subunit Coupling: Alternating Charged and Uncharged Achiral Linkages

In some applications, it may be desirable to introduce one or more charged achiral subunit linkages in the polymer molecules. The backbone charge may be used to enhance polymer solubility in many solution applications or to adjust polymer-target binding constants, as will be described further below. Polymers having regularly spaced charged achiral backbone linkages, e.g., between every second or third subunit, are suitable for the novel diagnostic system which is described below. For purposes of the present discussion, it is assumed that polymeric units composed of two or more subunits which are internally joined by achiral uncharged linkages are to be linked by a charged achiral linkage. These uncharged units are formed by one of the methods described in Sections A and B above.

The dimer subunits which are to be linked are selected such that that the free N_2 group of one dimer can be coupled to the free N_1 group of the other dimer through a suitable charged achiral linkage. The preferred linkage is a phosphodiester linkage which requires that the free N_2 group on one subunit and the free N_1 group on the other subunit both be hydroxyls. As can be seen in Figure 3, this condition is met if dimers formed from either structure A or structure B subunits, which both have a free N_2 hydroxyl, are linked to dimers formed from structure A, C, or D subunits which all have a free N_1 hydroxyl.

In a typical coupling method, dimer A, which is protected at its N_1 position, as indicated, is carried through a series of reactions which lead to an reactive phosphoester coupling species linked to the N_2 hydroxyl through a phosphoester bond, as indicated

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in Figure 9. The preferred coupling method, which is
detailed in U.S. patent application for "Polynucleotide
Assay Reagent and Method", Serial Number 712,396, filed
March 15, 1985, is detailed in Examples 23 and 24 below,
5 with respect to the formation of a tetramer have
alternating uncharged, stereospecific methylphosphonate
bonds, and charged achiral phosphodiester bonds.
Briefly, the illustrated method involves first forming
nucleotide dimers linked by methylphosphonate bonds,
10 isolatating stereoisomeric forms of the dimers and
activating one of the stereoisomeric dimers to form a
reactive N_1-protected N_2-(p-chlorophenyl-2-
cyanoethyl)-phosphate. The activated dimer is then
reacted with the second dimer to form a tetramer which
15 is linked alternately by uncharged, stereoisomeric
linkages and charged achiral intersubunit linkages.
    The phosphodiester linkage method may be
incorporated, either is solution or support-type polymer
synthesis method, according to the general procedures
20 outlined in Sections A and B for polymer coupling
involving achiral, but uncharged linkage reactions.   As
indicated, the phosphodiester bonds can be used to
couple dimeric or polymeric units which are themselves
coupled by uncharged achiral bonds, formed as above, or
25 for coupling dimeric units linked by uncharged, chiral
but-stereoisoeric linkages, such as the separated
stereoisomeric forms of methylphosphonate-linked dimers.
    For the diagnostics applications described
below, it is generally preferred to introduce as few
30 charged linkages as are necessary for achieving adequate
polymer solubility in an aqueous medium. The solubility
enhancement is generally achieved with 1-3 charged
backbone linkages per polymer of about 20 subunits, and
preferably no more than about one charged linkage per

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four subunits. As defined herein, achiral backbone linkages are considered substantially uncharged if they contain less than about one charge backbone linkage per dimeric unit, and preferably one or fewer backbone
5 linkage per tetrameric unit.

IV. Polymer Targeting Considerations

The design considerations applied in preparing
10 a polynucleotide binding polymer for use in the invention, are governed by the nature of the target analyte and the reaction conditions under which the analyte is to be assayed. As a first consideration, there is selected a non-homopolymeric target base
15 sequence against which the polymer is directed. This target sequence is preferably unique to the analyte being assayed, i.e., is calculated to occur only with some defined small probability (such as 1% or less) in an assay mixture containing a given number of unique-
20 sequence bases. The probability of occurrence of a given n-base target sequence is approximately $1/4^n$—that is, a given n-base target sequence would be expected to occur approximately once in a polymer containing 4^n bases. Therefore, the probability P
25 that a given n-base sequence will occur in polynucleotides containing a total of N unique-sequence bases is approximately $P=N/4^n$. To illustrate, the probability P that a 9-base target sequence will be found in a 20 kilobase polynucleotide is about $20 \times 10^3 / 2 \times 10^5$ or
30 0.08, the probability that a 16-base target sequence will be present is about $20 \times 10^3 / 4.3 \times 10^9$ or 0.0000047. From these calculations, it can be seen that a polymer having 9-16 recognition moieties specific for a defined 9-16 base target sequence should have high

specificity for the target sequence in an assay mixture containing only viral genomes, whose greatest complexities correspond to about 400K of unique-sequence bases.

Similar types of calculations show that a 12 to 5 16 subunit polymer can provide adequate specificity for a viral or bacterial target sequence in an assay mixture containing viral and bacterial genomic material only (largest genomic sizes about 5,000 kilobases), and that a 16 to 22 subunit polymer can provide adequate 10 specificity for a target sequence in a polynucleotide mixture containing mammalian genomic DNA material (genomic sizes of about 5 billion base pairs of unique-sequence DNA).

The polymer/analyte binding affinity, and 15 particularly the temperature at which the polymer just binds with the target sequence (the melting temperature, or Tm) can be selectively varied according to (a) polymer length, (b) the number of hydrogen bonds that can be formed between the recognition moieties and the 20 corresponding, in-sequence bases of the analyte target sequence, and (c) backbone charge density. From a number of studies on model homopolymer duplexes, it is known that the melting temperature of oligonucleotide duplexes in the 10 to 20 bp range increases roughly 3°C 25 per additional base pair where the complementary bases are capable of forming two hydrogen bonds, and about 6°C per additional base pair where the complementary bases are capable of forming three hydrogen bonds. Therefore, the length of a target sequence, which is initially 30 selected to insure high binding specificity with the polymer, may be extended to achieve a desired melting temperature with the complementary-base polymer, under selected assay conditions. Also, where the recognition moieties used in constructing the polymer are the

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standard nucleic acid bases, as illustrated above, the target sequence may be selected to have a high percentage of guanine plus cytosine bases for achieving a relatively high polymer/analyte melting temperature,

5 or a relatively high percentage of adenine plus thymine bases, for achieving a relatively low melting temperature.

The backbone charge density of the polymer must be substantially less than that of the polynucleotide

10 analyte, to allow preferential binding of polycationic reporter molecules to the analyte, under conditions where reporter binding to the polymer does not occur, as already noted. This requirement is met where the spacing between the adjacent negative charges in the

15 polymer backbone is at least twice as great as the spacing between adjacent charged phosphodiester linkages in the analyte. The charge density of the backbone also has an important effect on the polymer/analyte melting temperature, particularly under low salt conditions,

20 where any charge-charge repulsion between the analyte and polymer backbones can act to lower the temperature at which the two can anneal. In general, therefore, a polymer having a less-charged backbone would be expected to show (1) a higher analyte/polymer melting

25 temperature, and (2) less dependence of the melting temperature on salt concentration. Based on these considerations, an uncharged achiral polymer, such as the several polymers described above with respect to Figures 6 and 8, will allow the analyte/polymer

30 annealing reaction in the diagnostic method to be carried out under a wider range of salt-concentrations than a partially charged polymer, such as a polymer formed from alternating charged achiral phosphodiester

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bonds and uncharged linkages which may be either achiral
or chiral, bu stereospecifically defined.

V. Polymer Assembly Methods

5

A. Geometric assembly
          After selecting a desired polymer length and
recognition moiety sequence, according to factors
considered in Section IV, the polymer is assembled,
10   using the general subunit coupling procedures detailed
above. One method of polymer assembly involves initial
preparation of an appropriate set of dimers, linking
selected dimers to form tetramers, linking of these to
form octamers, and so on. This method is carried out in
15   solution, substantially according to methods described
with reference to Figures 5A and 7A above. It should be
noted that all couplings need not necessarily be between
oligomers of equal size. For example, often it is
desirable in the last coupling to link a hexadecamer
20   with a tetramer to give a 20-mer or to link a
hexadecamer with an octomer to give a 24-mer.
          A particular merit of this assembly method is
that each coupling product is roughly twice the mass of
the precursors and so purification of the product of
25   each coupling is a simplified matter. Example 18 below
details the the assembly of an 8-subunit
carbamate-linked polymer formed by this method.(b)

B. Stepwise Assembly on a Solid Support
30   One preferred method for polymer synthesis is
stepwise assembly on a solid support. Here the first
subunit in the polymer is attached through its N_2
backbone group to a solid support. Typically, a solid
support, such as glass beads derivatized with cleavable,

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preferably acid-stable, long-chain linkers are employed as the support material, and prepared for attachment of N_2 nucleophile, such as a 5' hydroxyl of a nucleoside, as descibed in Example 19. The glass beads are reacted

5   with a subunit which has a preferably acid-labile N_1 protected group, and an activated N_2 or E backbone group, as detailed in Section III above. The coupling of various types of subunits to a glass-bead support is described generally in Examples 20-22.

10   After coupling the second subunit (or polymer unit which may be assembled in solution) to the support, any unreacted linker nucleophiles are capped by addition of a suitable capping reagent, such as p-nitrophenyl acetate, and thereafter the support is washed and

15   filtered. The protecting group on the N_1 terminal subunit is removed, typically by acid treatment, and after neutralization, the support is then reacted with an excess of the next-in-sequence subunit (or polymer unit) which is activated at its free N_2 backbone

20   moiety. The excess of activated subunit maximizes the number of support-bound subunits which are chain-elongated. That is, one feature of the solid support assembly method is the need for high coupling efficiencies at each subunit addition step, and the

25   concentration of added activated subunit is selected to maximize this efficiency. Chain elongation is continued in this manner, with capping of failure sequence, after each subunit addition, until the polymer of the desired length and sequence is achieved. The general method is

30   illustrated in Example 20 for the preparation of a 14-subunit carbonate-linked polymer, in Example 21, for the synthesis of a 19-subunit carbamate-linked polymer, and in Example 22, for the assembly of a 19-subunit amide-linked polymer.

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After addition of the last-in-sequence subunit,
the polymer may be capped with a suitable charged or
uncharged group coupled to the terminal N_1 group. If
the backbone assembly has been carried out exclusively
5 with uncharged achiral linkages, the capping reagent is
preferably a charged group which imparts an end terminal
charge to the polymer. After capping the polymer, the
polymer is cleaved from the support, e.g., by treatment
with a nucleophile, such as ammonium hydroxide, and the
10 polymer is purified, such as by anion exchange
chromatography. The assembled, purified polymers are
attached to a solid support, by methods to be considered
below.

The polymer design principles discussed above
15 are illustrated in Examples 18 for a solution synthesis
from preassembled dimeric units, in Examples 20-23 for
the preparation of a variety of uncharged achiral
linkages, and in Example 24, for the preparation of an
alternating charge type polymer.

20

VI. Diagnostic Reagent

A. Solid Support Reagent
The reagent of the invention is formed by
25 coupling multiple binding polymers from above to a solid
support. Alternatively, the polymers may be synthesized
in a stepwise or block fashion, on the support, as
described above for the carbamate-linked polymers. The
polymers may be coupled directly to (or formed directly
30 on) the support, e.g., through an OH end group on the
polymer to an OH-reactive group on a solid support, such
as activated agarose, cellulose, or the like. However,
direct coupling often places the support-proximal
subunits too close to the support to allow base-

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complementary binding between the analyte and the
proximal subunit recognition moieties. Therefore, the
polymer molecules preferably are each linked to the
solid support through a spacer arm adapted to distance
5 the polymer from the surface region of the support so as
to allow substantially unhindered binding between the
polymer and analyte target sequence.

  The spacer arm is preferably an unbranched
chain having a total chain length of at least 6 atoms,
10 and has suitable reactive end groups for attachment to
the support, at one end, and to the polymer at the other
end. A variety of types of spacer arms, particularly
carbon-containing chains of various lengths and
hydrophilicity, are well known, as are reactive groups
15 used in spacer-arm coupling reactions. Example 25 below
describe the synthesis of an aminohexyl spacer arm, its
attachment to the 5' end of mixed
methylphosphonate/phosphodiester polymer, and coupling
to a solid support. Example 26 below details the
20 preparation of a polymer support having multiple
surface-bound, linear-chain spacer arms, for use in
forming a diagnostic reagent having carbamate-linked
polymers. The method of Example 26 is illustrated in
Figure 10. The support shown in the figure is an
25 aminomethylated polystyrene which is first reacted with
succinic anhydride to form a carboxylated derivative.
Activation of the support with di(succinimido)carbonate
and reaction with 6-aminohexanol leads to the 13-atom
terminal-hydroxyl spacer arm shown.

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Preferred solid supports include agarose,
cellulose, nitrocellulose, latex, polystyrene, and other
commercially available strip or particle-bead support
material having surface-reactive or activatable groups
5 which allow efficient coupling of polymer molecules,
particularly through polymer-bound spacer arms. The
concentration of polymers coupled to the support surface
is preferably selected so as to allow a 10-1000 fold
molar excess of polymer molecules to analyte molecules
10 when a suitable sample volume of assay material is mixed
with the support, in the diagnostic procedure, as will
be described below. A procedure for coupling a target
binding polymer to agarose beads through the aminohexyl
spacer arm from Example 25 is described in the same
15 example.

B. Reporter
        The reporter of the diagnostic system of the
invention is composed of two parts: (1) a polycationic
20 moiety or tail designed to bind electrostatically to a
fully charged polynucleotide, under conditions where the
reporter does not bind to the less charged binding
polymer carried on the diagnostic reagent, and (2) one
or more reporter groups attached to the tail adapted to
25 produce a signal by which the presence of the reporter
can be detected. Polycationic, as the term is used
herein, encompasses tails having two or more suitably
spaced cationic groups.
        The cationic tail is preferably constructed to
30 provide at least two positively charged groups which are
spaced for binding to adjacent negative charges on the
sugar-phosphate backbone of the polynucleotide analyte.
At the same time, the spacing between adjacent charges
on the cationic tail is such that electrostatic binding

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to the reagent polymer backbone which involves more than
half of the reporter charges is energetically
unfavorable. For example, in the above-described
polymer having alternating uncharged phosphonate and
5   charged phosphate linkages, the distance between
adjacent negative charges in the polymer backbone is
approximately twice that in a polynucleotide backbone.
Therefore, binding between substantially all of the
charged groups of the cationic tail and the alternately
10 spaced negative charges of the polymer backbone would
require energetically unfavorable contortion of the
polymer backbone. It can be appreciated that where the
polymer backbone has an alternating charged/uncharged
configuration, the spacing between adjacent positive
15 charges in the cationic tail should be close to the
minimum spacing which provides strong electrostatic
binding between the reporter tail and the polynucleotide
backbone. The charge-spacing requirement in the
reporter becomes much less critical, of course, where
20 the reagent polymer has a density of negative charge
less than one per two subunit linkages or where the
reagent polymer has an uncharged backbone.

It is also noted that when the polymer backbone
has an alternating charged/uncharged configuration, such
25 as charged phosphodiester linkages alternating with
uncharged methylphosphonate linkages, the cationic
reporter can most effectively discriminate between the
fully charged polynucleotides and the polymer when its
cationic moieties are unable to hydrogen bond to the
30 uncharged linkage, such as to the double-bonded oxygen
of the methylphosphonate linkage. For this reason, it
is generally advantageous to use quaternary ammonium
ions for the cationic groups in such a reporter.

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The number of cationic moieties in the reporter
tail may vary from two up to about 8 or more, according
to the total electrostatic attraction required to bind
the reporter to the polynucleotide analyte under binding
5 conditions where the reporter does not bind to the
reagent polymer backbone. For reporters having
relatively large reporter groups, such as enzymes,
multiple electrostatic binds may be required to attach
the reporter tail to the analyte backbone. A reporter
10 tail having a number of spaced cationic moieties is
particularly suitable for use with a reagent whose
polymer backbone is uncharged and thus cannot form
electrostatic bonds to the tail. Methods for
constructing a cationic tail suitable for use in the
15 invention are outlined in Figures 11-13. With reference
first to Figure 11, an N-methyl amino acid is
N-protected with di-t-butyl dicarbonate (BOC), activated
with carbonyl diimidazole, then coupled through an amide
linkage to dimethylamine. After deprotection, the amide
20 is reacted with an N-protected, carbonyl diiamidazole-
activated amino acid from above, to form a diamide
compound which is shown at the center in Figure 11. The
deprotection reaction and reaction with N-protected
diimidazole-activated amino acid, are repreated until
25 the desired-length polyamide is formed. Details of the
synthetic reactions are set forth in Examples 28-30.

A reporter group is most readily attached,
typically, to a primary amine group in the polycation.
To attach a primary amine to the secondary-amine end of
30 the Figure 11 compound, according to one suitable
method, an amino acid, e.g., 4-amino butyric acid, is
BOC-protected, activated with diimidazole, and reacted
with the deprotected polyamine, as illustrated in Figure
12. The resulting BOC-protected compound can then be

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reduced, after treatment with trifluoroacetic acid to
remove the BOC group, by reaction with borane-tetra-
hydrofuran.   These procedures are described in Examples
31-32.

5   Figure 13 shows a reaction scheme for
converting polyamine, such as synthesized above, to a
polyquaternary ammonium salt.   The procedure involves
protection of the terminal 1° amine followed by reaction
with methyl iodide, to form the poly quaternary ammonium
10 salt, and deprotection with trifluoroacetic acid.
Example 33 below gives details of the method.

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Preferred solid supports include agarose,
cellulose, nitrocellulose, latex, polystyrene, and other
commercially available strip or particle-bead support
material having surface-reactive or activatable groups
5   which allow efficient coupling of polymer molecules,
particularly through polymer-bound spacer arms. The
concentration of polymers coupled to the support surface
is preferably selected so as to allow a 10-1000 fold
molar excess of polymer molecules to analyte molecules
10   when a suitable sample volume of assay material is mixed
with the support, in the diagnostic procedure, as will
be described below. A procedure for coupling a target
binding polymer to agarose beads through the aminohexyl
spacer arm from Example 25 is described in the same
15   example.

B. Reporter
         The reporter of the diagnostic system of the
invention is composed of two parts: (1) a polycationic
20   moiety or tail designed to bind electrostatically to a
fully charged polynucleotide, under conditions where the
reporter does not bind to the less charged binding
polymer carried on the diagnostic reagent, and (2) one
or more reporter groups attached to the tail adapted to
25   produce a signal by which the presence of the reporter
can be detected. Polycationic, as the term is used
herein, encompasses tails having two or more suitably
spaced cationic groups.
         The cationic tail is preferably constructed to
30   provide at least two positively charged groups which are
spaced for binding to adjacent negative charges on the
sugar-phosphate backbone of the polynucleotide analyte.
At the same time, the spacing between adjacent charges
on the cationic tail is such that electrostatic binding

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to the reagent polymer backbone which involves more than
half of the reporter charges is energetically
unfavorable. For example, in the above-described
polymer having alternating uncharged phosphonate and
5 charged phosphate linkages, the distance between
adjacent negative charges in the polymer backbone is
approximately twice that in a polynucleotide backbone.
Therefore, binding between substantially all of the
charged groups of the cationic tail and the alternately
10 spaced negative charges of the polymer backbone would
require energetically unfavorable contortion of the
polymer backbone. It can be appreciated that where the
polymer backbone has an alternating charged/uncharged
configuration, the spacing between adjacent positive
15 charges in the cationic tail should be close to the
minimum spacing which provides strong electrostatic
binding between the reporter tail and the polynucleotide
backbone. The charge-spacing requirement in the
reporter becomes much less critical, of course, where
20 the reagent polymer has a density of negative charge
less than one per two subunit linkages or where the
reagent polymer has an uncharged backbone.

It is also noted that when the polymer backbone
has an alternating charged/uncharged configuration, such
25 as charged phosphodiester linkages alternating with
uncharged methylphosphonate linkages, the cationic
reporter can most effectively discriminate between the
fully charged polynucleotides and the polymer when its
cationic moieties are unable to hydrogen bond to the
30 uncharged linkage, such as to the double-bonded oxygen
of the methylphosphonate linkage. For this reason, it
is generally advantageous to use quaternary ammonium
ions for the cationic groups in such a reporter.

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The number of cationic moieties in the reporter
tail may vary from two up to about 8 or more, according
to the total electrostatic attraction required to bind
the reporter to the polynucleotide analyte under binding
5   conditions where the reporter does not bind to the
reagent polymer backbone. For reporters having
relatively large reporter groups, such as enzymes,
multiple electrostatic binds may be required to attach
the reporter tail to the analyte backbone. A reporter
10   tail having a number of spaced cationic moieties is
particularly suitable for use with a reagent whose
polymer backbone is uncharged and thus cannot form
electrostatic bonds to the tail. Methods for
constructing a cationic tail suitable for use in the
15   invention are outlined in Figures 11-13. With reference
first to Figure 11, an N-methyl amino acid is
N-protected with di-t-butyl dicarbonate (BOC), activated
with carbonyl diimidazole, then coupled through an amide
linkage to dimethylamine. After deprotection, the amide
20   is reacted with an N-protected, carbonyl diiamidazole-
activated amino acid from above, to form a diamide
compound which is shown at the center in Figure 11. The
deprotection reaction and reaction with N-protected
diimidazole-activated amino acid, are repreated until
25   the desired-length polyamide is formed. Details of the
synthetic reactions are set forth in Examples 28-30.
A reporter group is most readily attached,
typically, to a primary amine group in the polycation.
To attach a primary amine to the secondary-amine end of
30   the Figure 11 compound, according to one suitable
method, an amino acid, e.g., 4-amino butyric acid, is
BOC-protected, activated with diimidazole, and reacted
with the deprotected polyamine, as illustrated in Figure
12. The resulting BOC-protected compound can then be

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reduced, after treatment with trifluoroacetic acid to
remove the BOC group, by reaction with borane-tetra-
hydrofuran. These procedures are described in Examples
31-32.

5   Figure 13 shows a reaction scheme for
converting polyamine, such as synthesized above, to a
polyquaternary ammonium salt. The procedure involves
protection of the terminal 1° amine followed by reaction
with methyl iodide, to form the poly quaternary ammonium
10   salt, and deprotection with trifluoroacetic acid.
Example 33 below gives details of the method.

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The one or more reporter groups in the reporter
should be (1) readily attached to the cationic tail, and
(2) capable of generating an easily detected signal,
either when the reporter is bound to a polynucleotide
5 backbone, or after the reporter has been eluted from the
analyte. Small reporter groups that include chromo-
phores, such as nitroaniline or other strongly absorbing
dyes, and fluorophores, such as dansyl or rhodamine-
based fluorescent molecules, are suitable and have the
10 advantage that they can be readily detected by photo-
metric equipment, or in the case of dyes, by visual
inspection. Radioisotopic reporter groups may provide
advantages in diagnostic sensitivity, but reporter
detection may be more complex and expensive. Stable
15 paramagnetic molecules, such as nitroxide spin labels,
may also be used, the binding of the reporter to the
polynucleotide being detected by broadening of
electronic spin resonance (esr) absorption lines
characteristic of immobilized paramagnetic species.
20   Another class of suitable reporter groups
include ligand molecules, and preferably small antigenic
molecules capable of binding specifically and with high
affinity to anti-ligand molecules. Exemplary ligand/
anti-ligand pairs include antigen/antibody, lectin/
25 carbohydrate, and biotin/avidin. The anti-ligand
molecule is part of a signal-producing binding conjugate
which also includes a signal-producing group, such as a
chromophore, fluorophore, or enzyme by which the
presence of ligand groups can be detected, when ligand/
30 anti-ligand binding has occurred.
The reporter may have enzyme reporter moieties,
particularly, as noted above, where the reporter tail
has more than two cationic groups. Representative
classes of enzymes are oxidoreductases, typified by

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luciferase; glucose oxidase; galactose oxidase and
catalase; hydrolases, typified by various kinds of
phosphatases; glycosyl hydrolases, such as
β-galactosidase; peptidases; and lyases.

5   Typically the reporter group or groups are
coupled to an amine of the polycationic tail, and
preferably a primary amine, according to known coupling
methods. In one preferred method, illustrated in Figure
15, a polyamine is reacted with a suitable bifunctional
10 coupling agent, such as 4-fluoro-3-nitrophenylazide, and
then coupled to a reporter group, such as an enzyme. A
variety of bifunctional reagents for coupling amines to
reactive reporter groups such as amine, carboxyl, OH,
and sulhydral groups, are well known. A detailed method
15 for forming a tetracationic reporter with an alkaline
phosphatase reporter group is given in Example 38. In
another preferred procedure, illustrated in Figure 15, a
reporter is prepared by reacting bis-3,3'-aminopropyl-
methylamine with an amine-reactive dansyl group. The
20 reaction scheme in the figure also indicates how the
amino moieties of the reporter tail can be fully
alkylated before or following reporter coupling to the
tail. Details of the procedure are given in Example 34.

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VII. Diagnostic Method

This section describes the use of the
diagnostic system described above for determination of a
5 polynucleotide analyte. The analyte may be one which
normally exists in a single-stranded form, such as
messenger RNA (mRNA), ribosomal RNA (rRNA) or a single-
stranded RNA or DNA viral genome, or one which exists
under physiological conditions in a double-stranded or
10 duplex form, such as double-stranded viral RNA, RNA/DNA
viral replicative intermediates, and duplex DNA, such as
that derived from a virus, a bacterium, or eukaryotic
cell. The considerations and rationale used in selecting
a target sequence in the analyte polynucleotide, against
15 which the reagent polymers are targeted, are considered
in Section I.

In performing the diagnosis, a sample to be
assessed for analyte is collected, typically by conven-
tional clinical sample-handling techniques. Where the
20 analyte is a virus or bacteria-derived polynucleotide,
it is often useful to first process the sample to remove
larger non-analyte cell material. For example, in
assaying for the presence of a viral pathogen, it is
often useful to filter the sample through a 0.2 micron
25 pore-sized membrane to remove bacterial material. In
assaying for the presence of a bacterial pathogen, it is
often useful to filter the sample through a 1.0 micron
pore-sized membrane to remove eucaryotic cells, which
may also be present in the sample. Typical sample
30 preparation methods are described for diagnosis of viral
pathogens in Examples 36 and 37.

To prepare the sample analyte material in a
form suitable for binding to the reagent polymer, the
sample should be treated to free the organism's nucleic

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acid. For organisms lacking cell walls, detergents and/or chaotropic salts are generally adequate. For organisms having cell walls, alkali (when the analyte is DNA) or suitable enzymes (e.g., lysozyme for many

5   bacteria) can be used. If desired, the freed nucleic acids can be conveniently separated from proteins and other contaminants by addition of a chaotropic salt (sodium trichloroacetate) followed by selective precipitation of the nucleic acid with ethanol.

10   One efficient method for freeing and isolating nucleic acid material from viral or cell components has been described by the inventor in Anal Biochem (1983) 133:79. The method, which is detailed in Example 36, involves suspending the sample in 4M trichloroacetate,

15   100 mmol EDTA, to denature and solubilize proteinaceous material, then precipating freed nucleic acid material in the salt solution by the addition of an equal volume of cold ethanol. A carrier polynucleotide, such as polyuridylic acid, may be added to facilitate nucleic

20   acid precipitation. After a short chilling time, the precipitated nucleic acid is pelleted by centrifugation, and washed with aqueous ethanol to remove salt.

          The nucleic acid fraction is resuspended in annealing buffer having a selected salt concentration

25   and a divalent cation chelating agent. An annealing buffer containing about 100 mmol or less of monovalent salt, such as NaCl, and about 10 mmol chelating agent, such as EDTA, is generally suitable for binding of a polynucleotide which exists normally in a duplex DNA

30   form. Salt concentration significantly below 100 mmol may be difficult to achieve with precision. Higher salt concentrations, particularly above about 0.5 M, tend to allow duplex formation between complementary polynucleo- tide strands, leading to competition between the reagent

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polymer and the complementary strand for binding to the analyte polynucleotide. Duplex formation between complementary polynucleotides is also inhibited by the chelating agent, which acts to sequester Mg^{+2} and
5   Ca^{++} ions in the annealing mixture. Where the analyte is an RNA, it may be advantageous to treat the sample with DNase to prevent competition between dissociated DNA and the RNA analyte for binding to the reagent polymer.

10   The annealing reaction is preferably carried out at a temperature which is about 5°C to 15°C below the melting temperature of the analyte/polymer duplex structure which forms during the reaction period. This annealing temperature favors faithful base-sequence
15   pairing between the analyte target sequence and the polymer. As indicated above, where the analyte polynucleotide normally exists with its complementary strand as a duplex structure, the reaction temperature is preferably higher than the melting temperature of the
20   native polynucleotide duplex, to avoid undesired pairing of the analyte with its complementary polynucleotide competing with the desired pairing of the analyte with the reagent polymer. At an annealing buffer concentration of about 100 mmol monovalent cation, annealing
25   temperatures in the range of 24° to 60°C are generally suitable. As noted earlier the actual optimal annealing temperature is a function of the length of the binding polymer, its ratio of A + T to C + G recognition moieties, and, for partially charged polymers, the salt
30   concentration.
         Depending on the structural characteristics of the polymer, as discussed above, the melting temperature of the analyte/polymer duplex structure may be substantially higher than a preferred reaction

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temperature, such as 37°C, in the particular annealing
buffer employed, such as 100 mmol monovalent salt. In
such cases, it may be convenient to lower the melting
temperature of the polymer/analyte duplex structure to
5   the desired temperature by the addition of a denaturant
such as formamide. To determine the amount of formamide
needed to achieve the desired melting temperature, the
melting curve of the polymer and the target sequence,
which may be a synthetic oligonucleotide having the
10   analyte target sequence, is determined by conventional
means, and at a number of different denaturant
concentrations, and, in the case of added formamide,
typically ranging between about 5% and 50% by volume of
denaturant. From this determination, the amount of
15   formamide needed in the annealing buffer in order to
achieve a melting temperature about 5° to 15° above the
desired reaction temperature is determined.

         The analyte sample in the reaction buffer is
added to the diagnostic reagent, preferably under
20   conditions where the binding polymers of the reagent are
present in a 10-1000 molar excess over the molar
concentration of analyte molecules in the assay
mixture. The polymer/polynucleotide annealing reaction
is carried out at the selected reaction temperature for
25   a period sufficient to allow substantial polymer/analyte
annealing, typically between 10 minutes and 3 hours.
The solid-support reagent is washed one or more times to
remove unbound material.

         Reporter is then added to the washed reagent
30   under preselected conditions to bind reporter molecules
to the fully charged backbone of the reagent-bound
analyte. The spatial/charge characteristics of the
reporter, which permit selected binding of the reporter
to the polynucleotide backbone have been discussed

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above. One important factor, where the reagent polymer has some negative backbone charges, is the salt concentration of the binding medium. At very low salt concentrations, electrostatic interactions are stronger,

5   and this may lead to undesirable binding of the reporter to the widely spaced anionic groups of the reagent polymer. In contrast, at very high salt concentrations, shielding of electrostatic charges may prevent desired binding of the reporter to the closely spaced anionic

10 groups of the analyte backbone. The optimal salt concentration of the binding medium is the maximum or near-maximum concentration of monovalent salt which permits adequate electrostatic binding of the reporter to the fully charged analyte backbone. This optimal

15 salt concentration can be determined by equilibrium dialysis techniques, in which binding of the diffusable reporter to a nondiffusable polynucleotide is measured as a function of salt concentration of the suspending medium. Optimal ionic-strength conditions for reporter

20 binding to the analyte may also be determined readily by binding the reporter to immobilized polynucleotide, under low salt conditions, and eluting the reporter with a salt gradient.

The binding components in the diagnostic

25 system, as they function in the diagnostic method of the invention, are shown below in Figure 16. Here "S" is the solid support having a number of binding polymers attached to its surface through spacer arms indicated by sawtooth lines, and the "m" and "p" subunit linkages

30 represent uncharged, stereoisomerically defined methylphosphonate and charged phosphodiester linkages, respectively. The reporter has a dicationic tail and an R reporter group. For illustrative purposes, each polymer has the sequence of recognition moieties

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complementary to the selected 16-base target sequence in
Herpes simplex virus, Types I and II, as described in
Example XXI. The base-complementary binding of one of
these polymers to the target sequence in a Herpes
5 simplex analyte polynucleotide is shown.

    The reporter shown in the figure is the
dicationic reporter from Example 34. Each reporter is
adapted to bind through electrostatic attraction to a
pair of adjacent phosphodiester bonds in the
10 polynucleotide through electrostatic attraction with
adjacent phosphodiester bonds. The reporter molecules
are shown in their expected binding configuration, where
substantially every phosphodiester linkage is paired
with a reporter cation, and the reporter molecules are
15 arrayed head-to-head and tail-to-tail. Assuming a
maximum density of bound reporter molecules, it can be
appreciated that an N-base analyte can bind up to about
N/2 reporter molecules. More generally, depending on
the size of analyte and the relative number of both
20 reporter moieties and cationic groups per reporter, the
assay method readily leads to binding of thousands to
several hundred thousand or more reporter molecules to
each reagent-bound analyte molecule. The sensitivity of
detection can therefore be between 2 and 4 orders of
25 magnitude greater than in existing types of
polynucleotide-based diagnostics, where analyte
detection is generally based on one or a few probe
molecules per analyte molecule, with each probe
typically containing roughly 100 reporter moieties.
30   After reaction with the reporter solution,
typically at room temperature for 1-2 minutes, the
reagent is washed to remove unbound reporter, and then
can be assessed directly for bound reporter. In
determining the amount of reporter associated with the

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reagent, it may be desirable, particularly in the case
of fluorescent or chromophoric reporter groups, to elute
the reporter from the reagent with a high salt solution
and then assess the eluate for reporter. Other types of
5   reporter groups, such as enzymes, can be readily
assessed with the reporters either bound to or eluted
from the reagent. Methods for determination of a
variety of different reporter groups, such as those
mentioned above, are well known. Examples 36 and 37
10   illustrate diagnostic procedures based on determination
of fluorescent and enzymatic reporters, respectively.

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From the foregoing, it can be appreciated how
various objects and features of the invention are met.
The diagnostic reagent can be readily tailored, by
reagent polymer design, for the detection of any ribo-
5 or deoxyribopolynucleotide having a unique base pair
sequence. Further, the reagent polymer can be tailored
to bind a given target sequence with high sequence
specificity at a convenient temperature.

For detection of duplex polynucleotides, the
10 reaction can be carried out under low-salt and/or
denaturing conditions which prevent competition from
complementary polynucleotide strands for analyte binding
to the reagent. This diagnostic system therefore
provides the advantage of the prior art solid-support,
15 single-probe polynucleotide diagnostic system, in that
competition between complementary strands is eliminated,
but avoids the rather laborious step associated with
that system in attaching single-strand test nucleic
acids to the solid support. At the same time, the
20 system avoids the problems associated with the
dual-probe polynucleotide diagnostic system described
earlier, in that analyte detection is based on
pseudo-first order kinetics, and only one
sequence-specific binding polymer is required.
25 According to another important feature of the
invention, reporter binding to the analyte is based on
sequence-independent electrostatic interactions rather
than sequence-specific binding, as in existing types of
polynucleotide diagnostics. Accordingly, the reporter
30 itself can be prepared relatively cheaply, can react
with the analyte quickly and under a wide range of
conditions, and, most importantly, can bind to the
analyte at a density ranging up to multiple reporter
moieties per polynucleotide subunit in the analyte.

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Accordingly, the sensitivity of the diagnostic system can be in the range of 2 to 4 or more orders of magnitude greater than existing tests, which rely on detection of one or a few probes, containing a limited

5 number of reporter moieties, per analyte molecule.

The following examples illustrate preparation and use of assay systems constructed according to particular embodiments of the invention, but are in no

10 way intended to limit the scope of the invention.

Example 1
Preparation of ribonucleosides and deoxyribonucleosides

15   The following nucleosides are obtained from Sigma Chemical Co., St. Louis, MO: deoxyuridine, deoxyguanosine, thymidine, deoxyadenosine, deoxycytidine, 5-bromodeoxyuridine, deoxyinosine, 2,6-diamino-9-(2-deoxy-ß-D-erythro-pentofuranosyl)9H-purin

20 e (2,6-diaminopurine deoxyriboside), uridine, guanosine, 5-methyluridine, adenosine, cytidine, 5-bromouridine, inosine.

       2,6-Diamino-9-(ß-D-ribofuranosyl)-9H-purine (2,6-diaminopurine riboside) is obtained from Pfaltz and

25 Bauer, Inc., Division of Aceto Chemical Co., Inc., Waterbury, CT.

       The following nucleosides are prepared by the literature methods indicated:

       1-(2-Deoxy-ß-D-erythro-pentofuranosyl)-2-

30 pyrimidinone (2-hydroxypyrimidine deoxyriboside) is prepared by the method of P. Kohler, E. Volz, U. Sequin, and C. Tamm in _Nucleic Acid Chemistry_ (L.B. Townsend and R.S. Tipson, eds) John Wiley and Sons, Inc. (1976).

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1-(2-Deoxy-ß-D-erythro-pentofuranosyl)-4-methoxy-
2-pyrimidinone is prepared by the following procedure:
1-(3',5'-Di-O-benzoyl-2-deoxy-ß-D-erythro-pentofu
ranosyl)-4-methylthio-2-pyrimidinone (prepared as in D.

5   Cech and A. Holy, Collection of Czechoslov Chem Comm
(1977) 42:2246) is treated with 200 mL of 0.2M sodium
methoxide solution. The resulting solution is allowed
to stand overnight at room temperature. This solution
is then neutralized with Dowex 50X8 (H+ form) and

10 filtered; the residue is dissolved in water (100 mL)
extracted with ether (2.50 mL) and the aqueous phase
evaporated. The residue is an amorphous material which
isused directly in succeeding reactions.
2-Amino-9-(2-deoxy-ß-D-erythro-pentofuranosyl)-1,

15 9-dihydro-6H-purine-6-thione (deoxythioguanosine) is
prepared by the procedure of R.H. Iwamoto, E.M. Acton,
and L. Goodman, J Med Chem (1963) 6:684.
1-(ß-D-Ribofuranosyl)-2-pyrimidinone
(2-hydroxypyrimidine riboside) is prepared by the

20 procedure of U. Niedballa and H. Vorbruggen, J Org Chem
(1974) 39:3668.
1-(2-Deoxy-ß-D-ribofuranosyl)-4-methoxy-2-pyrimid
inone (2-hydroxypyrimidine deoxyriboside) is prepared by
the procedure of R. Wightman and D. Holy, Collection of

25 Czechoslov Chem Comm (1973) 38:1381.
2-Amino-9-(ß-D-ribofuranosyl)-1,6-dihydro-
6H-purine-6-thione (thioguanosine) is prepared by the
procedure of J.J. Fox, I. Wempen, A. Hampton and I.L.
Doerr, J Amer Chem Soc (1958) 80:1669.

30

Example 2

Preparation of Base-Protected Nucleotides

Dimethoxytrityl chloride, N-benzoyladenosine,
N-benzoyl-2'-deoxyadenosine, N-benzoylcytidine,

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N-benzoyl-2'-deoxycytidine and N-isobutyryl-2'-deoxyguanosine are obtained from Sigma Chemicals, St. Louis, Missouri. 9-Fluorenylmethoxycarbonyl chloride (FMOC chloride), trimethylchlorosilane, isobutyric anhydride, 4-nitrobenzoyl chloride and all organic solvents for reactions and chromatography are obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Silica Gel is obtained from EM Science, Cherry Hill, New Jersey.

2.1 Guanosine·

The N-2 9-fluorenylmethoxycarbonyl derivative is prepared by the procedure below which is general for the protection of nucleoside amino groups:

Guanosine (1 mmol) is suspended in pyridine (5 mL) and treated with trimethylchlorosilane (5 mmol). After the solution is stirred for 15 minutes 9-fluorenylmethoxycarbonyl chloride (5 mmol) is added and the solution maintained at room temperature for 3 hours. The reaction is cooled in an ice bath and water (1 mL) is added. After stirring for 5 minutes conc. ammonia (1 mL) is added and the reaction stirred for 15 minutes. The solution is evaporated to near dryness and the residue dissolved in chloroform (10 mL). This solution is washed with sodium bicarbonate solution (5 mL, 10%), dried over sodium sulfate and evaporated. The residue is coevaporated several times with toluene and the product chromatographed on silica gel using a gradient of methanol in methylene chloride (0-50%).

N-Isobutyrylguanosine is prepared by the method of Letsinger and Miller, J Amer Chem Soc (1969) 91:3356.

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N-2 Acetylguanosine is obtained by the method
of C.B. Reese and R.S. Saffhill, J Chem Soc Perkin Trans
(1972) 1:2937.

5   2.2 Deoxyguanosine
        The N-2 9-fluorenylmethoxycarbonyl derivative
is prepared by the method of J. Heikkla and J.
Chattopadhyaya, Acta Chem Scand (1983) B37:263.
        The N-2 acetyl derivative is obtained from
10 Research Plus Inc., Bayonne, N.J.

    2.3 Deoxyadenosine
        The N-6 2-(4-nitrophenyl)-ethoxycarbonyl
derivative is prepared by the method of F. Himmelsbach
15 and W. Pfleiderer, Tetrahedron Lett (1983) 24:3583.
        N-6 4-Nitrobenzoyl-2'-deoxyadenosine is
prepared using the procedure above for FMOC-guanosine
except that 4-nitrobenzoyl chloride is substituted for
FMOC chloride.
20   The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is prepared by the procedure for FMOC
guanosine except the 2-(phenylsulfonyl)-ethyl
chloroformate (obtained from N. Balgobin, S. Josephson
and B. Chattopadhyaya, Tetrahedron Lett (1981) 22: 3667)
25 is used as the acylating agent and N-methylimidazole or
pyridine is used as the solvent.

    2.4 Adenosine
        The N-6 2-(4-nitrophenyl)-ethoxycarbonyl
30 derivative is prepared by the method of F. Himmelsbach
and W. Pfleiderer, Tetrahedron Lett (1983) 24:3583.
        N-6 4-Nitrobenzoyladenosine is prepared using
the procedure above for FMOC-guanosine except that

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4-nitrobenzoyl chloride is substituted for FMOC
chloride.

The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is prepared by the procedure for FMOC
5 guanosine except the 2-(phenylsulfonyl)-ethyl
chloroformate (obtained from N. Balgobin, S. Josephson
and B. Chattopadhyaya Tetrahedron Lett (1981) 22:3667)
is used as the acylating agent and N-methylimidazole or
pyridine is used as the solvent.

10

2.5 Deoxycytidine

The N-4 2-(4-nitrophenyl)-ethoxycarbonyl
derivative is prepared by the method of F. Himmelsbach
and W. Pfleiderer, Tetrahedron Lett (1983) 24:3583.
15   The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is prepared by the procedure for FMOC
guanosine except the 2-(phenylsulfonyl)-ethyl
chloroformate (obtained from N. Balgobin, S. Josephson
and B. Chattopadhyaya Tetrahedron Lett (1981) 22:3667)
20 is used as the acylating agent and N-methylimidazole or
pyridine is used as the solvent.

2.6 Cytidine

The N-4 2-(4-nitrophenyl)-ethoxycarbonyl
25 derivative is prepared by the method of F. Himmelsbach
and W. Pfleiderer, Tetrahedron Lett (1983) 24:3583.
The N-6 2-(phenylsulfonyl)-ethoxycarbonyl
derivative is prepared by the procedure for FMOC
guanosine except the 2-(phenylsulfonyl)-ethyl
30 chloroformate (obtained from N. Balgobin, S. Josephson
and B. Chattopadhyaya Tetrahedron Lett (1981) 22:3667)
is used as the acylating agent and N-methylimidazole or
pyridine is used as the solvent.

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2.7 <u>2,6-diaminopurine riboside</u>

The N-2,N-6-bis(9-fluorenylmethoxycarbonyl)
derivative of 2,6-diaminopurine riboside is prepared by
the general procedure.

5   The N-2,N-6-bis(isobutyryl) derivative is
prepared by the general procedure.

2.8 <u>2,6-diaminopurine-2'-deoxyriboside</u>

The bis N-2,N-6-(9-fluorenylmethoxycarbonyl)

10   derivative of 2,6-diaminopurine-2'-deoxyriboside is
prepared by the general procedure.

2.9 <u>Thioguanosine</u>

The N-2 9-fluorenylmethoxycarbonyl derivative

15   of thioguanosine is prepared by the general procedure.

2.10 <u>2'-Deoxythioguanosine</u>

The N-2 9-fluorenylmethoxycarbonyl derivative
of 2'-deoxythioguanosine is prepared by the general

20   procedure.

<u>Example 3</u>

<u>Preparation of 5'-amino-2',5-dideoxyribonucleoside</u>
<u>subunits.</u>

25   Carbon tetrabromide, sodium azide,
p-toluenesulfonyl chloride (tosyl chloride), triphenyl
phosphine and 10% palladium on carbon are purchased from
Aldrich Chem Co. Lithium azide is obtained from Kodak
Laboratory and Specialty Chemicals.

30

3.1 <u>General Method</u>

The nucleosides employed in this example are
thymidine, N6-benzoyl-2'-deoxyadenosine,
N4-benzoyl-2'-deoxycytodine, and 2'-deoxyinosine,

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2-hydroxy-pyrimidine-2'-deoxyriboside and the
N2-N6-bisisobutyryl derivative of 2,6-diamino
purine-2'-deoxyriboside (see Example 1). The required
dried nucleoside (1 mmol) is weighed into a reaction
5 vessel containing triphenyl phosphine (1.01 mmol) and
lithium azide (5 mmol). After the solids are suspended
in DMF, carbon tetrabromide (1.0 mmol) is added to the
vessel. The solution is stirred at room temperature for
24 h. After quenching the reaction with methanol, the
10 solution is evaporated to dryness. The residue is
chromatographed on silica gel eluting with methanol/
chloroform mixtures to afford the desired 5'-azido-2',
5'-deoxynucleoside.

The 5'-azido nucleoside (1 mmol) is dissolved
15 in ethanol. Hydrogenation in the presence of 10%
palladium on carbon (catalytic amount) under a hydrogen
atmosphere (35 psi) occurs over 10 h. The solution is
filtered and evaporation under reduced pressure affords
a crude solid. The solid is purified by trituration
20 with the appropriate solvent.

3.2 5'-Azidouridine derivativ

The 5'-azido derivative of 5-bromo-2'-deoxy
uridine is prepared via the method described above. The
25 5'-azido-t-bromo-2'-deoxyuridine (1 mmol) is treated
with triphenyl phosphine (1.5 mmol) in pyridine at room
temperature for 2 h. Concentrated ammonia (1 mL) is
added to the reaction vessel. After 14 h the solvent is
removed under vacuum. The residue is dissolved in THF
30 and this solution is added to hexanes. The precipitate
is collected and the solid triturated with the
appropriate solvent to afford the 5'-amino-5-bromo-
2'-deoxyuridine.

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3.5 5'-Azido guanosine

An alternate preparation of
5'-amino-2',5'-dideoxyguanosine is to treat the
protected 2'-deoxyguanosine (protected at either the
5 N-2-acetyl or the N-2-FMOC derivative) (1 mmol) with
tosyl chloride (1.3 mmol) in pyridine at 0°C overnight.
The solution is evaporated to dryness and the residue is
dissolved in chloroform. The resulting solution is
washed twice with aqueous sodium bicarbonate and dried
10 over sodium sulfate. The solvent is evaporated and the
residue chromatographed on silica gel eluting with
methanol/chloroform mixtures. The resulting tosylate (1
mmol) is treated with sodium azide (6 mmol) in DMF at
80-100°C for a few h. The solvent is removed by rotovap
15 and the residue is chromatographed on silica gel eluting
with chloroform/methanol solvent mixtures. The azide is
reduced to the desired amine using the above procedure.

Alternate protecting groups for the base
nitrogens of 2'-deoxycytidine, 2'-deoxyadenosine,
20 2'-deoxyguanosine, and 2,6-diaminopurinedeoxyriboside
are 2-(phenylsulfonyl)ethoxycarbonyl for
2'-deoxycytidine and 2'-deoxyadenosine and the use of
the 9'-fluorenylmethoxycarbonyl (FMOC) to protect
2'-deoxyguanosine and 2,6-diaminopurine deoxyriboside,
25 as in Example 2.

Example 4

Preparation of 3'-amino-2',3'-dideoxyribonucleoside

subunits.

30 Thymidine is transformed to 5'-O-acetyl-3'-
azido-2',3'-dideoxythymidine and this is converted to
3'-azido-2',3'-dideoxyadenosine and 3'-azido-2',3'-
dideoxyguanosine via the methods of M. Imazawa and F.
Eckstein, J Org Chem (1978) 43:3044. The base moiety of

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the 3'-azidoadenosine is protected as either the benzoyl
or the 2-(phenylsulfonyl)ethoxycarbonyl derivative (as
shown in Example 1). N-2 of the 3'-azidoguanosine is
protected as either the acetyl or FMOC derivative (see
5 Example 1). The reduction of these 3'-azido functions
to the 3'-amino-2',3'-dideoxyribonucleosides is
performed as shown in Example 3.

Example 5

10 <u>Preparation of morpholino-type subunits derived from
ribonucleosides.</u>

Ammonium biborate, sodium m-periodate, sodium
cyanoborohydride, uridine, N4-benzoyl cytidine, and
N6-benzoyl adenosine are obtained from Sigma Chemical
15 Co. N2-isobutyryl guanosine is prepared by the general
method of Letsinger and Miller (<u>J Amer Chem Soc</u> (1969)
<u>91</u>:3356). 2-Phenyl-2-propanol and bis(p-nitrophenyl)
carbonate are obtained from Aldrich Chemical Co.

The morpholino derivative of uridine is
20 prepared by dissolving 1 mmol of uridine plus 2 mmol of
ammonium biborate, $(NH_4)_2B_2O_7$, in 5 ml of
water. While stirring in an ice bath protected from
direct light 1.2 mmol of sodium m-periodate in 5 ml of
water is added. After 90 min, 0.2 ml of 1,2-propanediol
25 is added and stirring is continued for 10 min at rt.
Thereafter, while stirring in a well-ventilated hood,
0.25 g of sodium cyanoborohydride dissolved in 3 ml of
water is added and the mixture stirred for 4 hr at rt.
Thereafter, the reaction mixture is reduced to an oil
30 under vacuum in a warm water bath, resuspended in a
minimal volume of methanol, layered on a silica gel
chromatography column, and the column eluted with
methanol/1% triethylamine. The morpholino product
elutes from the column as a sharp band moving

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significantly slower than the original ribonucleoside
and its oxidation products. The morpholino product has
a UV spectrum essentially identical to the parent
ribonucleoside, but its mass is lower by 17 daltons
5   (determined by mass spectral analysis using fast atom
bombardment activation).

The morpholino nitrogen is next protected by
reacting the product with 2-phenylisopropyl phenyl
carbonate. The required active carbonate is prepared
10 and reacted with the morpholino subunit essentially by
the methods of Sandberg and Ragmarsson (_Int J Peptide_
Protein Res (1974) _6_:111). Finally, if desired
(depending on the method to be used for subunit assembly
into polymers) the hydroxyl at the position
15 corresponding to the original 5' can be activated by
dissolving the N-protected subunit in a minimal volume
of dry dimethylformamide and adding 2 equivalents of
bis(p-nitrophenyl)carbonate plus 0.2 equivalent of
triethylamine, N-methylimidazole, or N,N-dimethylamino
20 pyridine. After 3 h at rt, the reactions mixture is
reduced in volume under vacuum in a warm water bath.
The thick syrup is resuspended in a small volume of
dichloromethane and layered on a silica gel column,
which is subsequently eluted with a mixture of
25 dichloromethane/ether (1:1 by vol) 0.2% in
N,N-dimethylaniline by volume. With this solvent
system, the activated product moves substantially slower
than p-nitrophenol and bis(p-nitrophenyl)carbonate but
much faster than the unreacted starting material. The
30 activated product is readily visualized on silica gel
TLC plates simply by exposing to ammonia fumes,
whereupon a yellow nitrophenolate ion is generated
within one to two minutes.

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Other ribonucleosides (bases protected where
necessary as in Example 2) are converted to their
corresponding morpholino derivatives by essentially the
same methods -- though varying amounts of methanol must
5 be added to effect dissolution of the protected
ribonucleosides before the initial periodate oxidation
step.

When the morpholino-type subunits are to be
coupled via thiocarbamate linkages, the preferred
10 base-protective groups for the common starting
ribonucleosides are: the phenylsulfonylethoxycarbonyl
moiety for cytidine and adenosine and the 9-fluorenyl
methoxycarbonyl moiety for guanosine.

Example 6

15 Preparation of 5'-acetic acid-2',5'-dideoxyribonucleo-
side subunits.

Triethylamine, pyridine/sulfur trioxide
complex, p-nitrophenol, dicyclohexylcarbodiimide, and
20 40% aqueous dimethylamine are obtained from Aldrich.

3'-O-tert-Butyldimethylsilyl-N-2-(phenylsulfonyl)
ethoxycarbonyladenosine (1 mmol), prepared as in Example
12.1, is dissolved in methylene chloride and
dimethylsulfoxide (10 mL, 1/1, v/v) at 0°C and
25 triethylamine (3 mmol) and the pyridine/sulfur trioxide
complex (3 mmol) is added. The mixture is stirred at
this temperature for 1 h, then washed with water, dried
over sodium sulfate, and evaporated under reduced
pressure. The residue is dissolved in dry pyridine (10
30 mL) and treated with
benzyloxycarbonylmethylenetriphenlyphosphorane (1.5
mmol) for 24 h at 37°C. The mixture is then evaporated
to dryness under reduced pressure and the residue
dissolved in methylene chloride, washed with dilute HCl

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and water, then the organic phases are dried over sodium
sulfate and solvent removed under reduced pressure. The
residue is chromatographed on silica using a gradient of
methanol in chloroform (0-20%).

5   The unsaturated ester (1 mmol) from the
previous paragraph is dissolved in ethyl acetate/ethanol
(40 mL, 1/1, v/v) containing cyclohexadiene (5 mL) and
10% palladium on charcoal (100 mg) and the suspension is
vigorously agitated under a nitrogen atmosphere for 5-24

10   h. The mixture is filtered and the solvents removed
under reduced.pressure. The residue is chromatographed
on silica gel using a gradient of methanol in chloroform
(5-50%).

          To a 0.2-0.5 M solution of the acid from the
15   previous paragraph in ethyl acetate (or methylene
chloride) p-nitrophenol is added in about 20% excess.
The calculated amount of dicyclohexylcarbodiimide is
added to the solution at 0°C. After 0.5 h the mixture
is allowed to come to room temperature and was held

20   there for 1 h. The dicyclohexylurea which separated is
filtered and washed with solvent. The combined filtrate
and washings are evaporated to dryness under reduced
pressure. The ester may be used directly for coupling
reactions or may be purified by short .column

25   chromatography on silica using an isopropanol in
chloroform gradient (0-50%).

          The analogous series of reactions may be
carried out on the other properly protected
2'-deoxynucleosides.

30   The active ester (1 mmol) is treated with 40%
aqueous dimethylamine (2 mmol) in methanol to form the
N,N-dimethylamide of the N-protected 3'-O-tert-butyl
dimethylsilyl-2',5'-dideoxyadenosine-5'-acetic acid.
The solvents are removed by evaporation under reduced

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pressure and the residue is desilylated by using the 2M
HF/1M tert-butylammonium fluoride (TBAF) reagent
described in B.L. Gaffney and R.A. Jones, <u>Tetrahedron</u>
<u>Lett</u> (1982) <u>23</u>:2257. To the 2M HF/1M tert-butylammonium
5   fluoride in pyridine (1 mmol of TBAF) is added the
deprotected nucleoside from the previous paragraph.
After stirring for 24 h the reaction is partitioned
between methylene and aqueous sodium bicarbonate. The
organic layer is washed with water, dried over sodium
10   sulfate, and evaporated to dryness. The residue is
purified by chromatography on silica using a gradient of
methanol in methylene chloride (5-25%).

Example 7

15   <u>Preparation of Purine and Pyrimidine Pyrrolidones</u>
Potassium t-butoxide, anisoyl chloride,
6-chloropurine, 10% palladium on charcoal, phthaloyl
chloride, 2-amino-6-chloropurine, 20% aqueous
tetraethylammonium hydroxide, 25% aqueous
20   trimethylamine, 2-pyrimidinone, N-bromosuccinimide,
trifluoroacetic acid, 4-nitrophenol, and
N,N-disuccinimydyl carbonate are obtained from Aldrich.
Lithium azide is obtained from Eastman Kodak, Rochester,
New York. C18 reverse phase silica gel is obtained from
25   Whatman, Hillsboro, Oregon.

7.1 <u>Preparation of (S)-5-[(4-amino-2-oxopyrimidinyl)-</u>
<u>methyl]-pyrrolidone (henceforth referred to as the</u>
<u>cytosine pyrrolidone)</u>
30   Cytosine (2.2 mmol) is dissolved in dimethyl
sulfoxide (2 mL) which contained potassium tert-butoxide
(2 mmol). This solution is added to a flask which
contained (S)-5-(tosyloxymethyl)-2-pyrrolidone (1 mmol,
prepared by the procedure of E. Hardegger and H. Ott,

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Helv Chim Acta (1955) 38:312). After dissolution, the
mixture is stirred at 25°C for 6 h. The mixture is
neutralized with acetic acid (2 mmol) and evaporated
under reduced pressure. The residue is taken up in
5   dimethyl formamide (5 mL) and evaporated under reduced
pressure, and this procedure repeated three times.

7.2 Preparation of N-4 anisoylcytosine pyrrolidone
        The mixture from the previous paragraph is
10 dissolved in pyridine or N-methylimidazole (10 mL) and
treated with anisoyl chloride (2.8 mmol) at room
temperature. After stirring for 2 h, the mixture is
quenched with ice (0.5 g). After 5 min, 1 mL of 29%
ammonia was added. After 15 min at rt the solution is
15 dissolved in ethyl acetate (15 mL) and washed twice with
brine (15 mL). . The washings are combined and washed
with ethyl acetate (2 x 30 mL), the combined organic
layers dried with sodium sulfate and evaporated under
reduced pressure. The residue is coevaporated several
20 times with toluene and the residue chromatographed on
silica gel with a gradient of methanol in methylene
chloride (0-20%).

7.3 Preparation of 6-chloropurine pyrrolidone
25   This compound is prepared as for the alkylation
of cytosine except that the alkylation is performed on
6-chloropurine and the mixture was heated at 35-95°C for
several h. The mixture is cooled to room temperature,
neutralized with acetic acid (2 mmol) and evaporated
30 under reduced pressure, shaken with a mixture of 20%
methanol/chloroform and 10% sodium bicarbonate. The
aqueous layer is washed with chloroform, the combined
organic layers aree dried over sodium sulfate and
evaporated under reduced pressure. The residue is

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chromatographed on silica gel using a gradient of methanol in chloroform (0-25%).

7.4 Preparation of 6-azidopurine pyrrolidone

The 6-chloropurine pyrrolidone (1 mmol) is treated with lithium azide (2 mmol) in dimethyl sulfoxide (2 mL) at 30-100°C for several h. At the end of this time the mixture is evaporated under reduced pressure, dissolved in chloroform, washed with 10% sodium bicarbonate, and the organic layer dried over sodium sulfate and concentrated under reduced pressure. The residue is chromatographed on silica using a gradient of methanol in chloroform (0-25%).

7.5 Preparation of adenosine pyrrolidone

The azide from the previous example is hydrogenated by shaking the azide (1 mmol) with 30 pounds of hydrogen pressure in a solution of ethanol (10 mL) containing palladium on charcoal (100 mg, 10% by weight Pd) for 24 h. The solution is filtered through celite and the solvent evaporated. The residue is used directly for the next step.

7.6 Preparation of N-6 phthaloyladenosine pyrrolidone

The phthaloyl group is introduced at the N-6 position by the acylation of the adenosine pyrrolidone (1 mmol) with phthaloyl chloride (1.4 mmol) using the procedure above for the anisoylation of cytidine except that no ammonia was added in the workup.

7.7 Preparation of 2-amino-6-chloropurine pyrrolidone

The pyrrolidone tosylate is alkylated with 2-amino-6-chloropurine as per the preparation of the 6-chloropurine derivative.

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7.8 Preparation of guanine pyrrolidone

The chloropurine pyrrolidone (1 mmol) from the previous paragraph is dissolved in diglyme (10 mL) and 25% aqueous trimethylamine (2 mL). The solution is stirred at rt for 5 h, water (10 mL) is added, and the mixture concentrated to 10 mL under reduced pressure. Acetic acid (2 mL) is added, and the mixture was evaporated under reduced pressure to an oil. The residue which contains tetraethylammonium acetate is used directly for the next step.

7.9 Preparation of N-2 acetylguanine pyrrolidone

Guanine pyrrolidone (1 mmol) from the previous paragraph is reacted with acetic anhydride (5 mL) at reflux for 2 h. The solvent is evaporated and the residue coevaporated with dimethylformamide. The residue is dissolved in methanol (5 mL) saturated with ammonia at 0°C, and the solution is stirred for 2 h at this temperature. The solvent is evaporated under reduced pressure and the residue purified by chromatography on a short column of silica using a gradient of methanol in chloroform (5-50%).

7.10 Preparation of 2-amino-6-azidopurine pyrrolidone

This compound is prepared from the 2-amino-6-chloropurine pyrrolidone by use of the procedure of Example 7.4, except that the reaction requires longer times and was performed at a higher temperature.

7.11 Preparation of 2,6-diaminopurine pyrrolidone

This compound is prepared as for the adenosine pyrrolidone by reduction of the 2-amino-6-azido derivative.

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7.12 <u>Preparation of N-2 acetyl N-6 phthaloyl 2,6-diamino</u>
<u>purine pyrrolidone</u>

    The acetyl group is introduced by the method
above for N-2 acetylguanine pyrrolidone except that the
5 methanolic ammonia treatment is carried out for 8 h.
The solvents are evaporated and the residue is
phthaloylated as for the adenosine derivative.

7.13 <u>Preparation of inosine pyrrolidone</u>

10   6-Chloropurine pyrrolidone (1 mmol) was
converted to the inosine derivative by the method used
for the preparation of the guanine pyrrolidone
derivative. The compound is purified by chromatography
on silica using a gradient of methanol in chloroform
15 (5-25%).

7.14 <u>Preparation of 2-hydroxypyrimidine pyrrolidone</u>

    This compound is obtained by the alkylation of
2-pyrimidinone by the procedure used for the preparation
20 of the 6-chloropurine derivative.

·<u>Example 8</u>

<u>Preparation of Purine and Pyrimidine T-BOC Amino Acids</u>

    The general procedure described below for the
25 cytosine pyrrolidone may be applied to all properly
protected pyrrolidone derivatives. The product,
referred to as the t-BOC acid is formed by cleavage of
the pyrrolidone ring to an acid and a t-BOC amine.

30 8.1 <u>Preparation of the cytosine t-BOC acid</u>

    N-4-Anisoylcytosine pyrrolidone (1 mmol) is
dissolved in methylene chloride (0.5 M solution)
containing di-tert-butyl dicarbonate (2 mmol),
triethylamine (1 mmol), and dimethylaminopyridine (1

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mmol). The solution is stirred for 10 h at rt. The
volatiles are removed and the residue purified on silica
using a gradient of methanol in chloroform (0-20%). The
residue is dissolved in tetrahydrofuran (0.2 M solution)
to which is then added lithium hydroxide (3 mmol, as a
1 M solution). After 5 h at rt, the solution is
neutralized by the addition of 10% acetic acid (3 mmol)
and the solvents removed under reduced pressure. The
residue was dissolved in 9N ammonia and stirred at room
temperature for 1-10 h. The solvent is removed under
reduced pressure and the residue purified by
chromatography on C18 reverse phase silica gel using
water and methanol (or trifluoroethanol) mixtures.

8.2 Preparation of uracil t-BOC acid
          This is prepared from the cytosine t-BOC acid
by the following procedure:
          The cytosine t-BOC acid (1 mmol) is dissolved
in aqueous acetic acid and treated with sodium nitrite
(1 mmol) at 4°C. The mixture is stirred for 1 h and
evaporated to dryness under reduced pressure. The
product is purified by chromatography in C18 reverse
phase silica gel using water and methanol (or
trifluoroethanol) mixtures.

8.3 Preparation of 5-bromouracil t-BOC acid
          This compound is obtained from the uracil t-BOC
acid by the following procedure:
          The uracil t-BOC acid (1 mmol) is dissolved in
DMF (3 mL) and treated with N-bromosuccinimide (1.2
mmol). The solution is allowed to stand at room
temperature for 16 h. After removal of the solvents
under reduced pressure the product is purified by

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chromatography on C18 reverse phase silica gel using
water and methanol (or trifluoroethanol) mixtures.

8.4 Preparation of N-protected t-BOC acid

5   This general procedure for acylating the
exocyclic amino groups of the recognition moiety is also
applicable to the protection of the adenosine derivative.
           The cytosine t-BOC acid (1 mmol) from the
previous paragraph is dissolved in N-methylimidazole or
10 pyridine (5 mL) and treated with chlorotrimethylsilane
(5 mmol). After 15 min at room temperature the solution
is treated with 2-(4-nitrophenyl)ethylchloroformate (3
mmol, obtained from F. Himmelsbach and W. Pfleiderer,
Tetrahedron Lett (1983) 24:3583). The reaction iss
15 maintained at room temperature for 8 h, then cooled in
an ice bath, and water (1 mL) was added. After 5 min, 1
mL of concentrated ammonia is added and the mixture
stirred at rt for 15 min. The reaction is then
evaporated to dryness and the residue dissolved in
20 water. The solution is made acidic with 2 M HCl and
extracted with chloroform. The combined organic
extracts are dried over sodium sulfate and evaporated
under reduced pressure. The residue is purified by
chromatography on silica using a gradient of methanol in
25 chloroform (5-50%).
           For protection of the guanine and
2,6-diaminopurine derivatives, the above procedure was
modified so that the reaction was carried out in
pyridine and the acylating agent was 9-fluorenylmethyl
30 chloroformate.

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Example 9

Preparation of Purine and Pyrimidine Amino Acids

The following procedure is applicable to all
the derivatives. It removes the t-BOC group and frees
5   the primary amino group of the backbone.

The t-BOC acid (1 mmol) was dissolved in 20%
trifluoroacetic acid in methylene chloride (5 mL) and
stirred at rt for 30 min. Toluene (3 mL) is added and
the solvents are evaporated under reduced pressure to
10 give the amino acid trifluoroacetate salt. This is used
directly for coupling reactions.

Example 10

Preparation of Subunits for Assembly

15 of Achiral Acyclic Polynucleotide Analogs

10.1   Preparation of the Cytidine Analog

Lithium borohydride, di-tert-butyl dicarbonate
and bis(triphenylphosphine) palladium II chloride were
20 obtained from Aldrich Chemical Co., Milwaukee, WI.
2-Amino-5-bromopyrimidine is prepared as per T.
Nishikawa, Chem. Pharm. Bull, 9:38 (1961).

2-amino-5-bromopyrimidine is benzoylated at the
exocyclic amino group by the general method in Example 2
25 using benzoyl chloride. The product benzamide (25
mmole) is added to a 45 mL pressure vessel containing
triethylamine (10 mL), benzene (10 mL), and
bis(triphenylphosphine) palladium II chloride (0.375
mmole). The bomb is flushed with argon, sealed, and
30 pressurized to 600 psi with carbon monoxide and then
pressurized to 1200 psi with hydrogen. The reaction
vessel is heated in an oil bath with stirring at 145°C.
after all gas absorption had ceased, the reaction is
cooled, and the gases vented slowly. After addition of

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anhydrous ether, the reaction mixture is filtered and
evaporated in vacuo. The product was isolated following
chromatography in silica gel using a gradient of
isopropanol in methylene chloride (0-20%).

5   The 2-benzamidopyrimidine-5-carboxaldehyde (1
mmol) from the previous paragraph is dissolved in
ethanol (5 mL) and treated with 4-aminobutyric acid (1
mmole) and triethylamine (1 mmol). After stirring for
one hour, the mixture is hydrogenated at 30 psi using

10   10% palladium on carbon (100 mg). After the absorption
of hydrogen has ceased, the reaction is filtered and the
solvents removed under reduced pressure. The residue is
dissolved in methanol which is saturated with ammonia at
0°C and stirred at room temperature for 10 h. After

15   evapoaration of the solvents, the residue iss purified
by chromatography on C18 silica gel using methanol water
mixtures buffered to pH 7 with ammonium acetate (0.2 M).
          The amino acid (1 mmol) from the previous
paragraph is dissolved in ethanol (3 mL) containing

20   di-tert-butyl dicarbonate (1 mmol). Triethylamine (1.5
mmol) is added slowly at room temperature. After the
evolution of carbon dioxide has ceased, the solvents are
removed in vacuo and the residue used directly for the
next reaction.

25   The t-BOC-amino acid from the previous
paragraph is acylated on the exocyclic amino group by
the general procedure in Example 2 using
2-(phenylsulfonyl)-ethoxycarbonyl chloride. The product
may be purified by chromatography on silica gel using a

30   gradient of methanol in chloroform (0-50%).
          The 2-(phenylsulfonyl)-ethoxycarbonyl-protected
BOC amino acid from the previous paragraph was converted
into the p-nitrophenyl or N-hydroxysuccinimidoyl active
ester by the procedures in Examples 11.3 and 11.4.

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10.2.   Preparation of the Uridine Analog

Ethyl 6-hydroxynicotinate is prepared as per H.
Gault, J. Gilbert, and D. Briaucourt, C.R. Acad. Sci.,
Paris, Ser. C, 266:131 (1968). Lithium borohydride is
5   obtained from Aldrich. Manganese dioxide is prepared as
per Vereshchagin et al, Zhurnal Arganicheskoi Khimil,
8:1129 (1972). Dowex resin is obtained from Bio-Rad
Laboratories, Richmond, CA.

Ethyl 6-hydroxynicotinate (49 mmole) in
10   tetrahydrofuran (10 mL) is added dropwise to a
suspension of·lithium borohydride (100 mmol) in
tetrahydrofuran (THF) (75mLO at room temperature under
nitrogen. The viscous mixture is stirred at 25°C for 16
hours. Then, 60 mL of 20% acetic acid is added to the
15   cooled suspension. The THF is evaporated under reduced
pressure and the remaining aqueous solution is applied
to an ion exchange column (Dowex-50W-X8) to remove
lithium ion. The column is washed carefully with 250 mL
of water, which is then evaporated. Boric acid is
20   removed from the resulting residue by repeated
evaporation with methanol. The product is purified by
distillation under reduced pressure.

The benzylic alcohol (66 mmol) prepared in the
previous paragraph is dissolved in ether (250 mL) and
25   treated slowly (exothermic) with a slurry of manganese
dioxide (54 g) in ether (100 mL). After twelve hours at
room temperature, the mixture is filtered, the solvent
removed under reduced pressure, and the residue
distilled in vacuo to give 6-hydroxynicotinaldehyde.
30   The aldehyde from the previous paragraph is
reacted with 4-amino butyric acid as for the preparation
of the cytidine analog in Example 11.1

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The amino acid from the previous paragraph was converted to the corresponding BOC-derivative as for the preparation of the cytidine analog in Example 10.1

The BOC-amino acid from the previous paragraph
5 iss converted into the p-nitrophenyl or
N-hydroxysuccinimidoyl active ester by the procedures in Example 11.3 and 11.4.

Example 11

10 Preparation of 5'-protected, 3'-activated
2'-deoxynucleo- sides.

11.1 2'-deoxynucleoside 5'-O-dimethoxytrityl
derivatives

15   The procedure below is general for the preparation of 2'-deoxynucleoside 5'-O-dimethoxytrityl derivatives:
N-2 9-Fluorenylmethoxycarbonyl-2'-
deoxyguanosine (1 mmol) is dissolved in anhydrous
20 pyridine (2.0 mL) and kept at 0°C. Dimethoxytrityl chloride (1.2 mmol) is added in portions (0.15 mmol) during an 8 hour period. After an additional 2 hours methanol (0.5 mL) was added and the solution is concentrated at reduced pressure. The residue is
25 treated with a mixture of sodium bicarbonate (2 mL, 5%) and methylene chloride (5 mL).
The aqueous layer is extracted with methylene chloride (2x5 mL) and the combined organic layers are dried over sodium sulfate and evaporated. The residue
30 is coevaporated several times with toluene and the product is purified by chromatography on silica gel using a gradient of methanol in methylene chloride (0-10%).

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11.2 5'-O-dimethoxytrityl-2'-deoxynucleoside
3'-O-(4-nitrophenyl)carbonates.

The preparation of 5'-O-dimethoxytrityl-2
-deoxycytidine 3'-O-(4-nitrophenyl)carbonates is
5   described in the following procedure which is general
for the preparation of the 4-nitrophenylcarbonates. Bis
(4-nitrophenyl)carbonate and N,N-dimethylaminopyridine
are obtained from Sigma.

5'O-Dimethoxytrityl-N-2-(4-nitrophenyl)-
10 ethoxycarbonyl-2'-deoxycytidine (1 mmol) is dissolved in
anhydrous dimethylformamide (5 mL) at room temperature
and treated with bis(4-nitrophenyl)carbonate (2 mmol)
and then evaporated under reduced pressure. The residue
is dissolved in dimethylformamide (5 mL) and
15 N,N-dimethylaminopyridine (0.1 mmol) is added. The
yellow solution is evaporated and allowed to react for 1
h. The reaction mixture is dissolved in chloroform (10
mL) and washed with aqueous HCl (5 mL, 0.1 M). The
organic layer is washed twice with aqueous NaOH (5 mL,
20 0.1 M), water (2 mL), dried over sodium sulfate and
evaporated. The residue is applied to a silica gel
column and eluted with a gradient of isopropanol in
chloroform (0-10%). The fractions containing the
product are pooled, evaporated, dissolved in chloroform
25 (10 mL) and the aqueous NaOH washes are repeated to
remove traces of 4-nitrophenol. The organic layer is
washed with water, dried over sodium sulfate and
evaporated. The product is homogeneous by TLC and is
used directly for preparation of the dimeric
30 carbonates.

For the preparations of the carbonates of
nucleosides containing the FMOC group it is sometimes
advantageous to use N-methylimidazole as the catalyst
rather than N,N-dimethylaminopyridine.

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11.3 N-protected t-BOC p-nitrophenyl ester

The following two procedures prepare active esters for coupling of subunits. It is general for all the t-BOC acid derivatives.

5   To a 0.2-0.5 M solution of the t-BOC acid from Example 8.4 in ethyl acetate (or methylene chloride), p-nitrophenol is added in about 20% excess. The calculated amount of dicyclohexylcarbodiimide is added to the solution at 0°C. After 0.5 h the mixture is

10   allowed to come to rt and was held there for 1 h. The dicyclohexylurea which separated is filtered and washed with solvent. The combined filtrate and washings are evaporated to dryness under reduced pressure. The ester may be used directly for coupling reactions or may be

15   purified by short column chromatography on silica using an isopropanol-in-chloroform gradient (0-50%).

11.4 N-protected t-BOC N-hydroxysuccinimidoyl ester

To a solution of the t-BOC acid from Example

20   8.4 (1 mmol) in acetonitrile (5 mL) is added pyridine (1 mmol) and N,N'-disuccinimidyl carbonate (1 mmol). The solution is stirred at rt for 3 h. The solvents are removed under reduced pressure and the residue dissolved in chloroform, the organic phase washed with 0.1 M HCl

25   and water, dried over sodium sulfate, and evaporated to dryness. The esters are sufficiently pure to be used directly.

Example 12
Formation of a carbonate intersubunit linkage.

30   The preparation of cytidinyl-(3'-5')-cytidine carbonate is described in the procedures below. This is a general procedure for the formation of the carbonate intersubunit linkage and the deprotection of the

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oligocarbonate. Note that the base protecting groups
must be 2-(4-nitrophenyl)ethoxycarbonyl, 2-(phenyl
sulfonyl)ethoxycarbonyl, or FMOC. Tert-butyl
dimethylsilyl chloride is obtained from Aldrich.

5

12.1 <u>Preparation of 2'-deoxynucleoside 3'-O-tert-butyldi-
methylsilyl derivatives.</u>

        The preparation of 3'-O-tert-butyldimethylsilyl-
N-2-(4-nitrophenyl)ethoxycarbonyl-2'-deoxycytidine is
10   described in the following procedure which is a general
procedure for the preparation of 2'-deoxynucleoside-3'-
O-tert-butyldimethylsilyl derivatives:

        To a stirred solution of 5'-O-dimethoxytrityl-
N-4-(4-nitrophenyl)-ethoxycarbonyl-2'-deoxycytidine
15   (1 mmol) and imidazole (3.5 mmol) in dry
dimethylformamide or tetrahydrofuran (10 mL) is added
dropwise a solution of tert-butyldimethylsilyl chloride
(3 mmol) in dry DMF (10 mL). The reaction is stirred
for 1 h, quenched with ice, extracted with methylene
20   chloride, washed with water and dried over sodium
sulfate. The organic layer is evaporated to dryness
under reduced pressure and purified by chromatography
over silica gel with methylene chloride as eluant.

        To a solution of the 5'-O-dimethoxytrityl
25   derivative from the previous paragraph (1 mmol) in
methylene chloride (5 mmol) is added a solution of zinc
bromide in methylene chloride (10 mL, 1 M) containing
methanol (15 % v/v). The reaction mixture is stirred
for 30 min, quenched with ice, and extracted with
30   methylene chloride. The organic layer is washed with
water, dried over sodium sulfate, and evaporated to
dryness under reduced pressure. The product is purified
by chromatography over silica gel using a gradient of
methanol in methylene chloride (0-10%).

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5'-O-dimethoxytrityl-N-2-(4-nitrophenyl)ethoxy
carbonyl-2'-deoxycytidine-3'-O-(4-nitrophenyl)carbonate
(1.5 mmol) and 3'-O-tert-butyldimethylsilyl-N-4-(4-
nitrophenyl)ethoxycarbonyl-2'-deoxycytidine (1 mmol) are
5 dissolved in dimethylformamide (5 mL) and the solvent
removed under reduced pressure. This is repeated two
times. The residue is redissolved in dimethylformamide
(5 mL) and treated with N,N-dimethylaminopyridine (0.5
mmol) or N-methylimidazole (1 mmol). The solvent is
10 removed by evaporation under reduced pressure and the
reaction allowed to sit overnight at rt. The residue is
dissolved in chloroform (20 mL) and washed with aqueous
HCl (2 mL, 0.1 M), water (2 mL), aqueous NaOH (2 x 2 mL,
0.2 M), water (2 mL), and the organic layer dried over
15 sodium sulfate and evaporated. The residue is purified
on silica gel using a gradient of isopropanol in
chloroform (0-30%). The isolated product is homogeneous
on TLC and by 400 MHz NMR.
        The residue is desilylated by using the 2 M
20 HF/1M tert-butylammonium fluoride (TBAF) reagent
described in B.L. Gaffney and R.A. Jones, Tetrahedron
Lett (1982) 23:2257. To the 2M HF/1M tert-butylammonium
fluoride in pyridine (1 mmol of TBAF) is added the
deprotected nucleoside from the previous paragraph.
25 After stirring for 24 h, the reaction is partitioned
between methylene chloride and aqueous sodium
bicarbonate. The organic layer is washed with water,
dried over sodium sulfate, and evaporated to dryness.
The residue is purified by chromatography on silica
30 using a gradient of methanol in methylene chloride
(5-25%).
        The 3'-hydroxydinucleoside carbonates are
converted to the 3'-(4-nitrophenyl)-carbonates by the
method in Example 11. These derivatives may be reacted

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with the 5'-hydroxyl of a nucleoside or oligonucleoside carbonate to prepare higher order oligonucleoside carbonates.

Example 13

Formation of a carbamate intersubunit linkage.

5   Bis(p-nitrophenyl)carbonate, p-anisoyldiphenyl methyl chloride, N,N-dimethylaniline (DMA), and triethylamine are available from Aldrich Chemical Co.

10   The required 5'-amino-2',5'-dideoxyribonucleoside (employing acetyl or benzoyl groups for base protection where necessary) (1 mmol) prepared in Example 3 above, is treated with p-anisoyl diphenylmethyl chloride (1.2 mmol) in

15   pyridine. After 12 h the solvent is evaporated and the residue is redissolved in chloroform. This solution is washed once each with aqueous NaHCO$_3$ and water, then dried over Na$_2$SO$_4$. After evaporation of the solvent, the residue is chromatographed on silica gel

20   eluting with chloroform/methanol/1% triethylamine mixtures.

          The 5'-tritylated aminonucleoside (1 mmol) is evaporated twice from DMF, then treated with bis-(p-nitrophenyl)carbonate (2 mmol) in the presence of

25   triethylamine (or N,N-dimethylaminopyridine, or N-methyl imidazole)(catalytic amount) using DMF as the solvent. After 3 h, the solvent is evaporated and the residue dissolved in chloroform. This solution is washed twice with 0.01 N aqueous NaOH, once with water, then dried

30   over Na$_2$SO$_4$. The solvent is removed by rotovap and the residue is chromatographed on silica gel eluting first with a chloroform/0.1% DMA mixture, then with a chloroform/methanol/0.1% DMA solvent system. The appropriate fractions are combined and evaporated to

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dryness.   The residue is dissolved in a minimum of THF
and this solution is added to an excess of hexanes.   The
precipitated activated nucleoside is collected by
filtration and dried under vacuum.

5   The required 5'-aminonucleoside (1.1 mmol), as
prepared in Example 3 above, is twice evaporated from
DMF. The activated nucleoside (1 mmol) is added to the
reaction vessel and the solids are dissolved in DMF.
The solution is concentrated to a small volume and

10   allowed to stand overnight. The solvent is completely
removed under vacuum and the resulting residue is
dissolved in chloroform. This solution is twice washed
with 0.01 N aqueous sodium hydroxide, once with water,
then dried over solid sodium sulfate. The solvent is

15   removed by rotovap, and the residue is chromatographed
on silica gel eluting with the appropriate
methanol/chloroform/1% triethylamine solvent system.
The fractions containing the dimer nucleoside are
combined, then evaporated to dryness. The residue is

20   dissolved in a minimum of THF and this solution is added
to hexanes. The precipitate is collected and dried
under vacuum.

Example 14

25 Formation of a thiocarbamate intersubunit linkage.
            N,N-dimethylaminopyridine (DMAP) is purchased
from Aldrich Chemical Co.
            Formation of the thiocarbamate intersubunit
linkage of the 5'-aminonucleosides prepared in Example 3

30   is achieved in the same manner as described above in
Example 13 with the following alterations in procedure.
The 5'-aminonucleosides employed have as base-protecting
moieties either 2-(phenylsulfonyl)ethoxycarbonyl (for
deoxycytidine and deoxyadenosine) or 9-FMOC (for

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guanosine and 2,6-diamino-2'-deoxyriboside) groups. The
preparation of these molecules are described above
(Example 3). Activation of the 5'-tritylaminonucleoside
(1 mmol) is achieved with p-nitrophenylchlorothioformate
5   (as prepared by G Hilgetag and r. Phillippson, Monatsber
Deut Akad Wiss Berlin (1964) 6:897) (1.2 mmol) in the
presence of triethylamine (2 mmol) and the DMAP
(catalytic amount) in DMF. The coupling step employs
this activated monomer (1 mmol) with the requisite
10 5'-aminonucleoside (1.1 mmol) using DMAP or N-methyl
imidazole as catalyst and DMF as the solvent. In this
aqueous workup of these steps, the 0.01 N aqueous NaOH
washes are omitted, employing in their place water
washes.

15

Example 15

Formation of carbamate and thiocarbamate intersubunit linkages between morpholino type subunits.

N-methylimidazole is purchased from Aldrich
20 Chemical Co.

The requisite dry, N'-protected, 5'-free
alcohol morpholino type nucleoside (employing acetyl or
benzoyl groups for base protection where necessary)
(1 mmol), prepared in Example 5 above is treated with
25 bis-(p-nitrophenyl)carbonate and triethylamine (or DMAP
or N-methylimidazole) (catalytic amount) in DMF under
anhydrous conditions. The solution is stirred for 3 h,
then evaporated to dryness. The residue is dissolved in
chloroform and the solution is washed twice with 0.01 N
30 aqueous NaOH, once with water, then dried over
Na_2SO_4. The solvent is removed by rotovap and the
residue is chromatographed on silica gel eluting with an
appropriate chloroform/isopropanol/0.1% DMA mixture.
The fractions containing the desired activated

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nucleoside are combined, evaporated, and the resulting
solid is dissolved in a minimum of THF. The THF
solution is added to an excess of hexanes and the
resulting precipitate is collected and dried.

5   The required 5'-free hydroxy, N'-free
morpholino type nucleoside (using the identical base
protecting groups listed above in this example) (1.1
mmol), after evaporation twice from DMF, is·treated with
the 5'-(p-nitrophenoxycarbonyl)morpholino subunit (1

10   mmol) in DMF. The volume of the reaction solution is
reduced under·vacuum to a small volume. If required, a
catalytic amount of N-methylimidazole or triethylamine
is added to the reaction vessel. The resulting solution
is allowed to stand overnight at rt. The remaining

15   solvent is evaporated, and the residue is dissolved in
chloroform. The chloroform solution is washed twice
with 0.01 N aqueous NaOH, once with water, and then
dried over Na_2SO_4. The solvent is removed by
rotovap and the residue is chromatographed on silica gel

20   eluting with chloroform/methanol solvent mixtures. The
fractions containing the desired dimer are combined,
then rotovaped to dryness. The residue is dissolved in
a minimum of THF and the solution is added to an excess
of hexanes. The precipitate is collected and dried

25   under vacuum.
            Morpholino subunits linked by thiocarbamate
moieties are prepared as related above in this example
with the following experimental alterations. The base-
protecting groups are either

30   1-(phenylsulfonyl)ethoxycarbonyl (for deoxycytidine and
deoxyadenosine) or 9-FMOC (for deoxyguanosine and
2,6-diamino-2'-deoxyriboside) groups. The preparation
of these molecules is described above (Example 5).
Activation of the N-protected morpholino nucleoside

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(1 mmol) is achieved with p-nitrophenylchlorothioformate
(1.2 mmol) in the presence of triethylamine (2 mmol) and
DMAP or N-methyl imidazole (catalytic amount) in DMF.
The coupling step employs this activated monomer (1
5   mmol) with the requisite N'-free morpholino nucleoside
(1.1 mmol) using DMAP or N-methyl imidazole as catalyst
and DMF as the solvent while warming the solution to
35-40°C. In the aqueous workup of these steps, the 0.01
N aqueous NaOH washes are omitted, employing in their
10 place water washes.

Example 16

Preparation of an ester subunit linkage.

N,N-dimethylaminopyridine and N-methylimidazole
15 are obtained from Aldrich.

The coupling of two subunits is performed by
reaction of the 5'-N,N-acetamide-3'-hydroxy derivative
with the with the 3'-silylated-5'-active ester. These
are prepared as in Example 6. The reagents (1 mmol
20 each) are coevaporated several times with
dimethylformamide, then dissolved in dimethylformamide
(5 mL) and treated with N,N-dimethylaminopyridine (0.2
mmol) or N-methylimidazole (1 mmol). The solvent is
removed under reduced pressure and the reaction allowed
25 to stand at room temperature for 2 h. The residue is
dissolved in chloroform and washed with dilute HCl,
water, and the organic phase dried with sodium sulfate
and solvents evaporated under reduced pressure. The
residue is purified by chromatography on silica gel
30 using a gradient of methanol in chloroform.

The chain may be elongated by desilylation as
in Example 6, and treatment of the resulting 3'-free
hydroxy compound with the active ester as in the
previous paragraph.

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Example 17
Formation of a t-BOC amide-linked dimer

A fully protected t-BOC N-hydroxysuccinimidoyl
ester or p-nitrophenyl ester prepared as in Example 11.3
5 and 11.4 (1 mmol) is dried by several coevaporations
with dry dimethyformamide. The protected amino acid
trifluoroacetate salt from Example 9 (1 mmol) is treated
similarly. The two components are separately dissolved
in dry dimethylformamide (2 mL), mixed, and treated with
10 diisopropylethylamine (1.0 mmol). The solution is
stirred at room temperature for 1 h, then the solvent is
removed by evaporation under reduced pressure, the
residue dissolved in chloroform and washed with dilute
HCl. The organic layer is dried over sodium sulfate,
15 evaporated to dryness under reduced pressure, and the
residue purified by chromatography on silica gel using a
gradient of methanol in chloroform (5-50%).
Alternatively the t-BOC dimer acid may be purified by
chromatography on C18 reverse phase silica gel using
20 water and methanol (trifluoroethanol) mixtures.

17.1 Preparation of the t-BOC dimer active ester
        The acid from the previous paragraph is
converted into the N-hydroxysuccinimidoyl ester or
25 p-nitrophenyl ester by application of the general
methods in Example 11.3 and 11.4.

17.2 Preparation of the t-BOC dimer amino acid trifluoro
acetate
30   The t-BOC dimer acid is treated with trifluoro
acetate as per the general procedure in Example 9.

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17.3 Preparation of a t-BOC tetramer acid

The t-BOC dimer active ester and the dimer acid trifluoroacetate are coupled to the t-BOC tetramer acid by the general procedure. Purification is best effected
5 by chromatography on C18 reverse phase silica gel using water and methanol (or trifluoroethanol) mixtures buffered to pH 7.0 with triethylammonium acetate. In this manner chains of any length may be prepared by coupling an active ester with a free amine component.

10

Example 18
Geometric assembly of an 8-subunit carbamate-linked polymer

DEAE cellulose is purchased from Sigma Chemical
15 Co. Preparative TLC plates are a product of EM Science purchased from VWR Scientific. HPLC equipment, columns, and supplies are available from Beckman Instruments, Inc.

The requisite carbamate-linked dimer (0.2 mmol) prepared as described in example 12, is treated with 4
20 mL of a 1/1/1 mixture of methanol/THF/glacial acetic acid at rt for 12 h. The solvent is removed under vacuum and the residue is taken up in a minimum of THF. The THF solution is added to a large volume of hexanes and the resulting precipitate is collected and dried.
25 The acetate salt is dissolved in a 4/1 THF/ethanol mixture and DEAE cellulose (0.8 mmol of the base) is added to the vessel. After stirring for 20 min, the heterogeneous mixture is filtered and the filtrate evaporated to dryness. The residue is dissolved in a
30 minimum of 4/1 THF/ethanol and this solution is added to hexanes. The solid is collected and dried. The precipitation procedure is repeated once.

The required N-5'-trityl dimer (0.1 mmol) (as prepared in Example 12) is evaporated twice from DMF,

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then treated with bis(p-nitrophenyl)carbonate (2 mmol) in the presence of triethylamine (or DMAP or N-methyl imidazole) (catalytic amount) using DMF as the solvent. after 3 h the solvent is evaporated and the residue

5 dissolved in chloroform. This solution is washed twice with 0.01 N aqueous NaOH, once with water, then dried over Na_2SO_4. The solvent is removed by rotovap and the residue is chromatographed on silica gel eluting with a chloroform/isopropanol or methanol/0.1% DMA

10 mixture. The appropriate fractions are combined and evaporated to dryness. The residue is dissolved in a minimum of THF and this solution is added to an excess of hexanes. The precipitated activated dimer is collected by filtration and dried under vacuum.

15   The 5'-amino dimer nucleoside prepared above (0.11 mmol) is twice evaporated from DMF. The activated dimer (0.1 mmol) is added to the reaction vessel and the solids are dissolved in DMF. The solution is concentrated to a small volume and allowed to stand

20 overnight. The solvent is completely removed under vacuum and the resulting residue is dissolved in chloroform. This solution is twice washed with 0.01 N aqueous sodium hydroxide, once with water, then dried over sodium sulfate. The solvent is removed by rotovap

25 and the residue is chromatographed on a preparative TLC plate eluting with the appropriate methanol/chloroform/1% triethylamine solvent system. The band containing the tetramer nucleoside is eluted and the solvent is evaporated. The residue is dissolved

30 in a minimum of THF and this solution is added to hexanes. The precipitate is collected and dried under vacuum.

        The following transformations are performed using the procedures enumerated above in this example.

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The requisite N-5'-trityl tetramer nucleoside (0.06
mmol) is treated with 1/1/1 THF/methanol/glacial acetic
acid, then purified to afford the 5'-aminotetramer
nucleoside. Another aliquot of the N-5'-trityl tetramer
5 nucleoside (0.05 mmol) is activated with bis(p-nitro
phenyl)carbonate and subsequently purified. The two
tetramers are coupled, worked up, and further
purification is achieved either by chromatography on a
preparative TLC plate or by HPLC chromatography. the
10 chromatographed material is isolated as a solid,
dissolved in a minimum of THF, and then precipitated
from hexanes. The octomer nucleoside is collected and
dried.

       The octamer is configured for further use as ʹ
15 .follows. The N-5'-trityl octomer nucleoside is treated
with 1/1/1 THF/methanol/glacial acetic acid and purified
employing the conditions listed above. The 5'-amino
octomer nucleoside (0.01 mmol) is treated with succinic
anhydride (0.02 mmol) in pyridine at room temperature
20 for two hours. The solvent is removed by rotovap and
the residue is evaporated several times from ethanol.
The residue is taken up in 3/1 THF/ethanol and added to
a 2/1 hexane/benzene mixture. The solid is collected
and dried. The N-5'-succinylated octamer nucleoside
25 (0.01 mmol) is treated with a 1/1 mixture of
pyridine/concentrated ammonia (1 mL). After standing
overnight the solvent is removed by rotovap and the
residue is evaporated from ethanol several times. The
crude solid is purified using reverse phase (RP-18) HPLC
30 chromatography. The elutants are evaporated and the
residue is taken up in DMSO and precipitated from a 1/1
hexane/benzene solvent mixture. The solid is collected
and dried.

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Example 19

Preparation of a support-bound cleavable linker suitable
for stepwise and stepwise/block assembly of polymers:
activation of support and attachment of linker.

5   Long-chain alkylamine-derivatized controlled
pore glass (Cat. No. 24875) is obtained from Pierce
Chemical Co. 2,2-sulfonyldiethanol and
dicyclohexylcarbodiimide are obtained from Aldrich
Chemical Co.

10   The amino groups of the glass support
(approximately 40 mmol per gram of support) are reacted
with succinic anhydride essentially by the method of
Matteucci and Caruthers (J Amer Chem Soc (1981)
103:3185) to give free carboxylic acid termini.

15   One-tenth mol of 2,2'-sulfonyldiethanol (60 % by weight
in water) is dried by mixing with three volumes of
dimethylformamide (DMF), reducing to a thick syrup under
vacuum in a warm water bath, adding a second quantity of
DMF, reducing to a thick syrup, and repeating the

20   process a third time. Dry DMF is then added to give a
final sulfonyldiethanol concentration of 2 M. A mixture
of 1 ml of the sulfonyldiethanol solution plus 0.2 g of
dicyclohexylcarbodiimide is added to 1 g of the dry
controlled pore glass support carrying carboxylic acid

25   residues and the slurry mixed by rocking or tumbling
(not stirring) overnight at rt. Thereafter, the glass
support is thoroughly washed with methanol and dried.

30

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Example 20

Preparation of a 14-subunit carbonate-linked polymer
targeted against a conserved sequence in the AIDS viral
genome: stepwise assembly on a solid support: addition
5 of the first subunit and chain extension.

     N-methylimidazole,
4-(N,N-dimethylamino)pyridine (DMAP), and
1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) are obtained
from Aldrich Chemical co. Methyl p-nitrophenyl
10 carbonate is prepared from methyl chloroformate and
p-nitrophenol.

     The 2'-deoxycytidine subunit(0.1 mmol)
prepared as in Example 4.2 (wherein the N4 carries a
p-nitrophenethoxycarbonyl moiety, the 5' oxygen carries
15 a di(p-methoxy)trityl moiety (DMT), and the 3' oxygen
carries a p-nitrophenoxycarbonyl moiety) is dissolved in
a minimal volume of dry tetrahydrofuran (THF) or
dimethylformamide and added to 0.5 g of thoroughly dried
controlled pore glass support carrying a cleavable
20 linker, prepared as in Example 19. Catalyst (either 1
mmol of N-methylimidazole or 0.1 mmol of DMAP) is
introduced and the slurry is mixed by rocking or
trembling (not stirring) for 2 hours. The slurry is
next filtered, and the solid washed thoroughly with THF.

25   Any unreacted hydroxyls are capped by adding
two ml of THF 1 M in methyl p-nitrophenyl carbonate and
0.5 M in DMAP and mixing for 20 min at rt. Thereafter,
the glass support is washed with THF and filtered.

     The dimethoxytrityl at the 5' terminus is then
30 removed by washing the support with dichloromethane
followed by treatment with 5 ml of 0.2 M dichloroacetic
acid in dichloromethane for 5 min at rt. The glass
support is next washed with dichloromethane and filtered.

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Subsequent subunits, prepared as in Example 1
and activated as in Example 2, are added in a like
manner and in the following order: G,A,T,A,A,C,A,T,T,
T,T,T,C, (G protected with the FMOC moiety, A and C
5 protected with the p-nitrophenethoxy carbonylmoiety).

Example 21
Preparation of a 19-subunit carbamate-linked polymer
targeted against a conserved sequence in the AIDS viral
10 genome. Stepwise assembly of oligomer blocks on a solid
support: addition of the first subunit and chain
extension.

The 5'-amino-2',5'-dideoxycytidine subunit
(0.2 mmol), prepared as in Example 3 (wherein the N4
15 carries a benzoyl moiety, the 5'-amine carries a
p-methoxytrityl moiety, and the 3' oxygen carries a
p-nitrophenoxycarbonyl moiety) is dissolved in a minimal
volume of dry THF and added to 0.5 g of dried controlled
pore glass support carrying a cleavable linker, prepared
20 as in Example 19. Catalyst (either 1 mmol
N-methylimidazole or 0.1 mmol of DMAP) is introduced and
the slurry mixed by rocking or tumbling for 2 h. The
slurry is next filtered, and the solid washed thoroughly
with THF.

25 The mono-p-methoxy trityl is removed by washing
with dichloromethane, followed by treatment with 5 ml of
0.2 M dichloroacetic acid in dichloromethane at rt for 1
min. The support is then washed with dichloromethane
and filtered. The 5' amino termini are converted to
30 their free amine form by a brief wash with 3 ml of THF
containing 1% by volume of diisopropylethylamine,
followed by washing with THF and filtration.

0.1 mmol of 3' p-nitrophenoxycarbonyl-activated
dimer having the sequence 5'-A-G-3', prepared as in

Example 13 (wherein the guanine N2 is protected with an
acetyl moiety and the adenine N6 is protected with a
benzoyl or a p-nitrobenzoyl moiety), is dissolved in a
minimal volume of dry THF and added to the glass support

5 and mixing is carried out for 2 h (no catalyst is added
for this and subsequent coupling steps). The slurry is
next washed with THF and filtered.

Any unreacted amine moieties are capped by
adding 2 ml of THF 2 M in p-nitrophenyl acetate and

10 mixing at rt for 20 min. Thereafter, the glass support
is washed with THF and filtered.

The mono-methoxytrityl at the 5' terminus is
removed with dichloroacetic acid, as before.

Subsequent activated dimeric subunits, prepared

15 as in Example 17 (wherein the exocyclic ring nitrogen of
cytosine is protected with a benzoyl moiety and the
exocyclic ring nitrogen of A is protected with a benzoyl
or a p-nitrobenzoyl moiety), and added in a like manner
and in the following order: A-T, C-A, T-A, T-T, T-T,

20 A-C, A-C, C-A (5' to 3').

Subunits having base exocyclic ring nitrogen
protective groups removable by strong nonnucleophilic
bases (e.g., p-nitrophenethoxycarbonyl or phenylsulfonyl
ethoxycarbonyl for A and C, and FMOC for G) can also be

25 used for assembling polymers by the procedure above.
However, polymers having these alternative protective
groups are generally deprotected by treatment with DBU
rather than by ammonium hydroxide.

30

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Example 22
Preparation of a 19-subunit amide-linked polymer
targeted against a conserved sequence in the AIDS viral
genome. Stepwise assembly of oligomer blocks on a solid
5 support: addition of the first subunit and chain
extension.

A cytosine-containing acyclic-backboned subunit
(0.2 mmol), prepared as in Example 11.3 (wherein the N4
of the cytosine carries a 2(p-nitrophenyl)ethoxycarbonyl
10 moiety, the amine of the backbone carries a
t-butoxycarbonyl moiety, and the carboxyl is in the form
of a p-nitrophenyl ester) is dissolved in a minimal
volume of dry THF and added to 0.5 g of dried controlled
pore glass support carrying a cleavable linker, prepared
15 as in Example 19. Catalyst (either 1 mmol of
N-methylimidazole or 0.1 mmol of DMAP) is introduced and
the support mixed for 2 h, filtered, and the solid is
washed thoroughly with THF.

The t-BOC is removed by washing the support
20 with dichloromethane followed by treatment at rt with 5
ml of 20% by volume trifluoroacetic acid in methylene
chloride for 30 min. The support is washed with
dichloromethane and filtered. The support-bound amino
terminus is converted to the free amine form by a brief
25 wash with 3 ml of THF containing 1% by volume of
diisopropylethylamine, followed by a THF wash.

The activated dimeric subunit (0.1 mmol) having
the sequence (amino terminus)-G-A-(carboxy terminus)
prepared as in Example 17 (wherein the N2 of guanine
30 carries an FMOC moiety, the N6 of adenine carries a
p-nitrophenethoxycarbonyl moiety, the backbone amino
terminus carries a t-BOC moiety, and the carboxy
terminus is in the form of a p-nitrophenyl ester) is
dissolved in a minimal volume of dry THF and added to

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the glass support and mixing is carried out for 2 h (no catalyst is added for this or subsequent coupling steps). The support is washed with THF.

Any unreacted amine moieties are capped by
5   adding 2 ml of 2 M p-nitrophenyl acetate in THF and mixing at rt for 20 min. Thereafter, the glass support is washed with THF.

The terminal t-BOC moiety is removed with TFA and the support neutralized, as described above.
10   Subsequent dimeric subunits, prepared as in Example 17, are added in a like manner in the following order: (C-terminus) T-A, A-C, A-T, T-T, T-T, C-A, C-A, A-C (N-terminus).

Subunits having exocyclic ring nitrogen
15   protective groups removable by good nucleophiles (e.g., benzoyl for C, benzoyl or nitrobenzoyl for A, and acetyl or isobutyryl for G) can also be used for assembling polymers by the above procedures. However, polymers having these alternative protective groups are generally
20   deprotected by treatment with ammonium hydroxide rather than by DBU.

Example 23
Preparation of Nucleoside
25   3'-O-(p-chlorophenyl-2-cyanoethyl)-phosphates
23.1 Preparing Nucleosides with a 3' protecting group

Thymidine, N-benzoyldeoxyadenosine, N-benzoyldeoxycytidine, N-isobutyryldeoxyguanosine and their 5'-O-(di-p-methoxytrityl) 5'-ODMT-nucleoside)
30   derivatives are obtained from Pharmacia P-L Biochemicals (Piscataway, NJ). β-benzoylpropionic acid and dicyclohexylcarbodiimide (DCCD), are obtained from commercial sources. ·

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A selected 5'-O-dimethoxytrityl nucleoside (1 mmol) is reacted with ß-benzoylpropionic acid (3 mmol) and DCCD (4 mmol) in 6 ml pyridine. The reaction mixture is stirred at 25°C for three hours, after which

5 1.5 ml water is added and the mixture stirred for 5 hours at 25°C. The reaction mixture is filtered, and after evaporation of the filtrate, the residue is freed of pyridine by several coevaporations with ethanol. The purified residue is taken up in ethyl acetate and

10 chromatographed on silica gel using ethyl acetate. The concentrated ethyl acetate eluate is treated with hexane to precipitate the resultant 3'-O-ß-benzoylpropionyl-nucleoside (3'-Oßß-nucleoside).

15 23.2 <u>Nucleoside Activation</u>

p-Chlorophenylphosphorodichloridate is obtained from Aldrich (Milwaukee, WI). Benzenesulfonic acid, tetrahydrofuran, triethylamine, and 3-hydroxypropane-nitrile are obtained from commercial sources.

20 A solution of p-chlorophenylphosphoro-ditriazolide is prepared by sequential reaction of 3 mmol triazole in 10 ml tetrahydrofuran with 3 mmol triethylamine and 1.5 mmol p-chlorophenylphosphoro-dichloridate. After a 10-minute reaction at 25°C, 1

25 mmol of a selected 5'-ODMT nucleoside in 2 ml dry pyridine is added to the reaction mixture. After standing at 25°C for up to two hours, the mixture is treated with a solution of benzenesulfonic acid (4 mmol) and triethylamine (4 mmol) in 3 ml anhydrous pyridine,

30 followed by addition of 3-hydroxypropanenitrile (2 mmol). The reaction mixture is concentrated to 4 ml under vacuum and kept at 25°C for up to 2 hours, then at 4°C for up to 24 hours. The reaction is terminated by pouring the mixture into 20 ml of 5% aqueous sodium

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bicarbonate at 0°C, extracting thoroughly with
chloroform, drying the extracts with sodium sulfate, and
removing the solvents under reduced pressure. The
5'-ODMT nucleoside 3'-0-(p-chlorophenyl-
5 (2-cyanoethyl))-phosphate product is obtained by
chromatography on silica gel using successive elutions
of ether, ethyl acetate, and tetrahydrofuran.

10   Example 24
Preparing a tetramer with alternating charged and
uncharged bonds
         Methylphosphonodichloridate is obtained from
Aldrich. Benzenesulfonyl tetrazolide is prepared
15 according to Stawinski, et al, Nucleic Acids Research
(1977) 4:353.

24.1 Dimer Synthesis
         A solution of methylphosphonoditriazolide is
20 prepared by reacting 1,2,4-triazole (3 mmol) and
triethylamine (3 mmol) in 20 ml anhydrous tetrahydro-
furan in the presence of methylposphonodichloridate (1.2
mmol) for 2-10 hours at 25°C. Following filtration, a
solution of a selected 5'-ODMT nucleoside (1 mmol) in 10
25 ml dry pyridine is added to the filtrate, and the
mixture is concentrated to 8 ml and allowed to stand at
25°C for up to 2 hours. To the solution is added
benzenesulfonyl tetrazolide (1.2 mmol) and the selected
3'-OBB nucleoside . After incubation for 2.5 hours at
30 25°C, the reaction is quenched by the addition of 20 ml
of 50% sodium bicarbonate at -78°C and extracted
thoroughly with chloroform. The solvent is dried with
sodium sulfate and evaporated. The reaction may also be
performed substantially as above beginning with the .

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3'-O-PO-(OpCP)(OCE)-protected nucleoside phosphate from Example II.

24.2 Separating Stereoisomeric Methylphophonate Dimers

The stereoisomeric dimers produced in the above section are separated by silica gel column chromatography, carried our at atmospheric pressure in glass columns with packed silica gel 60, using ethyl acetate:tetra-hydrofuran mixtures (0-100% tetrahydrofuran) or chloroform/methanol mixtures (0-20% methanol). The faster moving stereoisomer fractions are combined, and precipitated from the tetrahydrofuran solution by the addition of hexane. The slower-moving stereoisomer is not used further.

24.3. Phosphodiester/Methylphosphonate-linked Tetramer

First and second stereospecific methylphospho-nate dimers, each having a selected 2-base combination and 3' OBD and 5' ODMT protecting groups, are each prepared as in Examples 24.1 and 24.2. Mesitylenesulfonyl tetrazolide is prepared according to Stawinski, et al, Nuc Acids Res (1977) 4:353.

The 5' ODMT protecting group on the first dimer is removed to give the pure 5' hydroxy compound. The cyanoethyl group of the second dimer is removed by treatment of 1 mmol of the dimer with a solution of pyridine (8.5 ml), water (2.9 ml), and triethylamine (2.9 ml) for 1 hour at 25°C, and evaporating the solvents to give the 3' hydroxynucleo-tide.

The dimer condensation reaction is carried out by dissolving the 5'-OH dimer (1 mmol) and the 3'-hydroxynucleotide dimer (1 mmol) in 8 ml of pyridine

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and treating with mesitylenesulfonyl tetrazolide (3-6
mmol) for 3.5 hours at 25°C. The reaction mixture is
combined with 4 ml of cold 50% aqueous pyridine and
poured into 80 ml of aqueous sodium bicarbonate. The
5 reaction product is extracted thoroughly with chloro-
form, and after drying over sodium sulfate and
evaporation of the organic solvent, is purified on
silica gel using a methanol/chloroform mixture. The
eluted product is precipitated with hexane. Tetramers
10 possessing the 3'-O-PO-(OpCP)(OCE) protecting group may
be prepared by substantially the same method beginning
with a dimer possessing the 3'-O-PO-(OpCP)(OCE) group.

Example 25

15 Attachment of polymer to a solid support through a
linker arm

25.1 Preparation of a bi-functional hexane linker arm
        Dowex 50X pyridinium resin is obtained from
20 Bio-Rad (Richmond, CA), and 6-aminohexanol, dimethyl-
formamide and 3-hydroxypropanenitrile are obtained from
commercial sources. 6-(2-methylsulfonyl)-ethyl p-
nitrophenyl carbonate is prepared according to Eberle,
et al., Helvetica Chimica Acta (1975) 58:2106.

25   A bi-functional hexane linker arm 6-(2-(methyl-
sulfonyl)-ethoxycarbonylamino)-hexanol is prepared by
reacting 6-aminohexanol (1 mmol) with 2-(methylsulfonyl)
ethyl p-nitrophenylcarbonate (1 mmol) in 1 ml dimethyl-
formamide for up to 4 hours at 25°C. The reaction
30 mixture is poured into water, extracted thoroughly with
benzene, the benzene washed with water, and the organic
solvent then dried with sodium sulfate and evaporated to
give the carbamate alcohol, which is purified by silica

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gel chromatography using a methanol/chloroform solvent
mixture.

25.2 Preparation of an Oligonucleotide Analog
with a 5'-O-(6-aminohexyl) Linker Arm

5   Triethylammonium bicarbonate and
tetrabutylammonium fluoride are obtained commercially.
A fully protected oligonucleotide analog is prepared by
the methylphosphonate-dimer condensation scheme

10   described in Example VI. The oligonucleotide (1 mmol)
is dissolved in 10 ml of chloroform:methanol (7:3, v/v)
containing 2% benzenesulfonic acid, and kept at 0°C for
about 40 minutes. The reaction mixture is washed with a
5% sodium bicarbonate solution and then water. The

15   chloroform layer is dried over sodium sulfate, filtered,
and evaporated under reduced pressure. The material is
dissolved in chloroform, and the residue chromatographed
on silica gel using chloroform/methanol mixtures to give
the 5' hydroxy oligonucleotide compound.

20   A solution of 6-(2-(methylsulfonyl)-ethoxy-
carbonylamino)-hexyl methylphosphonyl triazolide (5
mmol) in pyridine (10 ml) is prepared by treating the
carbamate alcohol from Example 25.1 with methylphospho-
noditriazolide, prepared as described in Example 24.1.

25   To the solution is added the 5' hydroxy compound (1
mmol) from the previous paragraph and benzenesulfonyl
tetrazolide (4 mmol). The reaction is continued and
worked up as for Example 24.1

The residue is chromatographed on silica gel

30   using a chloroform/methanol solvent mixture. The
protected oligonucleotide (1 mmol) is treated with 4 ml
hydrazine hydrate in 14 ml of 20% acetic acid/pyridine
for 16-24 hours at 25°C. After evaporation, the residue
is treated with a solution containing 0.017 M

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tetrabutylammonium fluoride in 30 ml tetrahydrofuran/
pyridine/water (8:1:1, v/v/v) for 24 hours at 25°C. The
solution is then treated with 60 ml 50% concentrated
ammonium hydroxide in pyridine for 10 hours at 4°C. The
5 resulting oligomer is purified on a reverse-phase HPLC
column using an aqueous acetonitrile mixture in 0.1 M
ammonium acetate buffer, pH 5.8, as the chromatography
solvent.

10 25.3 Attachment of a 5'-O-(aminohexyl)-
Oligonucleotide Analog to a Solid Support
        Affi-gel 10 is obtained from Bio-Rad (Richmond,
CA). 10 ml of the packed gel is washed with isopropyl
alcohol (3 bed volumes), and then with ice-cold
15 deionized water (3 bed volumes). A 5'-O-(6-aminohexyl)-
oligonucleotide (150 mmol) is prepared as described in
Example 26. The oligonucleotide (150 M) in 5 ml 0.1
M sodium bicarbonate buffer, pH 8.0, is slurried with
the washed gel for 1-4 hours at 25°C. The mixture is
20 then treated with 1 ml 1 M ethanolamine:HCl buffer, pH
8.0, to cap any unreacted active esters on the gel. The
gel is washed with water until all the buffer has been
removed, and stored at 4°C in the presence of 0.02%
sodium azide.

25

Example 26
Preparation of the Reagent Support
        Aminomethylated polystyrene is prepared as
30 described by B.A. Mukhitdinova, E.E. Eighozin, and G.A.
Makhmudova, Izv Akad Nauk Kaz SSR., Ser Khim (1980) 48.
Disuccinimido dicarbonate and 6-aminohexanol are
obtained from Aldrich.

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Aminomethylated polystyrene (1 g. 3.0 meq/g) is suspended in water/acetonitrile (10 ml, 3:1 v/v) and treated with succinic anhydride (20 mmol) at 4°C. The pH of the solution is kept at 6.0 by the addition of 20%
5   NaOH. When the pH of the solution holds constant, the reaction is continued for 24 hours. The beads are filtered, washed with 2 N HCl, water (until the pH of the washings is neutral) and made anhydrous by repeated washings with dioxane.

10   The support (having bound acid groups) from the previous paragraph is reacted with disuccinimido carbonate (20 mmol) in acetonitrile (10 ml) for 24 hours at 25°C. The support is isolated by filtration and the support having bound succinimido ester is washed
15   thoroughly with acetonitrile.

The support from above is suspended in dimethylformamide (10 ml) and reacted with 6-amino-. hexanol (20 mmol) for 24 hours at 25°C. The support is isolated by filtration and the support having the bound
20   alcohol chain is washed thoroughly with dimethyl-formamide.

Example 27

Procedure for Coupling

25   Aminonucleosides to the Support

N-methylimidazole, tetrabutylammonium chloride, tetrabutylammonium fluoride, and imidazole are obtained from Aldrich. Bis (p-nitrophenyl) carbonate is obtained from Sigma (St. Louis, MO).

30   The supported alcohol (10 mmol) is reacted with a coupling reagent such as carbonyl diimidazole (50 mmol) in dimethylformamide (30 ml) for 3 hours at 25°C. The activated support is isolated by filtration and thoroughly washed with dimethylformamide. The support

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is resuspended in 30 ml of dimethylformamide and treated
with a selected polycarbamate polymer (50 mmol)
prepared as in Example 21. After 3 hours, the supported
alcohol is filtered and thoroughly washed with
5   dimethylformamide.

    A selected probe is specific for a target
sequence comprising residues 8441 to 8456 of the ORF-2
gene of ARV-2, the etiological agent of AIDS
(Sanchez-Pescador et al, Science (1985) 227:484, is
10 synthesized by coupling the gel with the following
5'-amino nucleotides (in order of earliest
introduction): C,T,G,C,T,C,C,C,A,C,C,C,C,A,T,C, where C,
G, and A stand for the
N-(β-(trimethylsilyl)ethoxycarbonyl) protected-
15 5'-amino-2',5'-dideoxy cytidine, -guanosine, and
adenosine, respectively, and T stands for 5'-amino-2',5'-
dideoxythymidine.

    The supported polycarbamate (10 mmol) is
suspended in acetonitrile (30 mmol) and treated with
20 tetrabutylammonium chloride (3 mmol per meq of
protecting group) and potassium fluoride dihydrate (4
mmol per meq of protecting group) and heated at 50°C for
12 hours. At the end of this time water (30 ml) is
added and the supported probe is thoroughly washed with
25 water. The protecting group may also be removed with
tetrabutylammonium fluoride in tetrahydrofuran (3 mmol
per meq of protecting group) under the same conditions.

Example 28

30 ## Protection of the Amino Group as the BOC-derivative
4-(N-tert-butoxycarbonyl-N-methylamino)-butyric Acid

    4-(Methylamino)-butyric acid is obtained from
Aldrich. Di-tert-butyl dicarbonate is obtained from
Pierce (Rockford, IL).

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4-(methylamino)-butyric acid (10 mmol) is
dissolved in dioxane/water (30 ml, 2/1) and 1 N NaOH (10
ml) is added. The solution is cooled to 0°C and
di-tert-butyl carbonate (11 mmol) is added with
5 stirring. After 30 minutes at 25°C, the dioxane is
removed under reduced pressure and the pH of the
solution is adjusted to pH-2.5 by the addition of
potassium bisulfate. The aqueous phase is thoroughly
extracted with ethyl acetate and the organic layers
10 combined and dried over sodium sulfate. Removal of the
solvents under reduced pressure gives the free acid
which is purified by recrystallization from
chloroform/hexane, or by chromatography on silica gel
using chloroform/methanol mixtures.

15

Example 29
Introduction of the Terminal Dimethylamino Group
and General Procedure for the Activation of the
Acid by Conversion to the Imidazolide and General
20 ### Procedure for Coupling with an Amine

N,N'-carbonyl diimidazole is obtained from
Aldrich.

A solution of the BOC-protected amino acid (or
oligopeptide acid, 1 mmol) is treated with carbonyl
25 diimidazole (1 mmol) at -20°C for 6 hours. At this
-time, a solution of dimethylamine (excess) or
oligopeptide amine (1 mmol) prepared as in Example 30,
in DMF (1 ml) is added at -20°C and the reaction then
stirred at 25°C for 12 hours. The solvent is removed by
30 reduced pressure and the residue is chromatographed on
silica gel using methanol/chloroform to give
N,N-dimethyl 4-(N-tert-butoxycarbonyl-N-methylamino)-
butyramide.

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Example 30

General Procedure for the Removal of the
BOC-protecting Group of
N,N-Dimethyl 4-(methylamino)-butyramide

5   Trifluoroacetic acid is obtained from Aldrich.

N,N-Dimethyl 4-(N-tert-butoxycarbonyl-N-methyl-amino)-butyramide (of BOC-protected oligopeptide, 1 mmol) (Example XV) is dissolved in trifluoroacetic acid (5 ml) and stirred for 1 hour at 25°C. The solvent is

10   removed and the residue is partitioned between 1 N NaOH/saturated NaCl (1/10) and chloroform. The aqueous phase was thoroughly extracted with chloroform and the combined organic layers were dried over sodium sulfate and evaporated under reduced pressure to give the free

15   amine. This could be used directly for further coupling reactions, or can be purified on silica gel using methanol/chloroform mixtures containing 1% triethylamine.

Using the coupling procedure and the BOC deprotection sequence, polyamides of various lengths may

20   be prepared. For example, reaction of 4-(N-tert-butoxycarbonyl-N-methylamino)-butyryl imidazolide with N,N-dimethyl 4-(methylamino)-butyramide produces the dimeric monoamide. Removal of the BOC group and coupling with another equivalent of the

25   imidazolide produces the trimeric diamide, the process being repeated until the desired length is achieved.

Alternatively, 4-(N-tert-butoxycarbonyl-N-methylamino)-butyryl imidazolide is reacted with 4-(methylamino)-butyric acid and the resulting dimeric

30   acid is activated with N,N-carbonyl diimidazole and coupled with free amino oligoamides.

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Example 31
General Procedure for the Reduction of
the Amide Linkages to Amines

AG-50 and Dowex-50W are obtained from Bio-Rad

5 (Richmond, CA).

The oligomeric polyamide to be reduced is
treated with trifluoroacetic acid as in the general
procedure in order to remove the BOC group. After
isolation and purification, the free amine (1 mmol) in

10 tetrahydrofuran (1 ml) is added dropwise to a solution
of borane (2 mmol per amide residue) in tetrahydrofuran
(3 ml) at 25°C. The colorless solution is refluxed for
1 hour, cooled to 0°C, and treated dropwise with 6 N HCl
(1ml). After all evolution of gas has ceased, the

15 solution is evaporated under reduced pressure to remove
the tetrahydrofuran, and the remaining aqueous solution
is applied to an AG-50 ion exchange resin and purified
by washing the column of water (10 ml) and eluting the
polyamine with a buffer comprised of sodium acetate

20 (0.1-2.0 M) and sodium chloride (0.1-2.0 M) at pH 5.
After collection and evaporation of the fractions
containing the desired product, the residue is dissolved
in 1 N HCl (4 ml) and applied to a Dowex 50W ion
exchange column. After washing with water, and then 2 M

25 HCl, the product is recovered as the hydrochloride by
elution with 6 N HCl and removal of the solvent under
reduced pressure.

The primary amino group of the polyamine is
protected as the BOC-derivative and converted to the

30 ammonium salt using methyl iodide as per Example 33.

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Example 33

General Procedure for the Incorporation of
a Terminal Primary Amino Group into the Polyamine

4-aminobutyric acid is converted to the
BOC-derivative. This is reacted with carbonyl
diimidazole as in Example 28 and treated wtih the
dimeric amide from Example 29. Cleavage of the
BOC-group and reduction as in Example 31 produced the
polyamine.

The primary amino group of the polyamine is
protected as the BOC-derivative as in the general
procedure and converted into the ammonium salt using
methyl iodide as per Example 33.

Example 33

General Method for the Conversion of the Polyamine
to the Poly (quaternary ammonium) Salt (3-aminopropyl-)-
dimethyl-(3-(5'-dimethylamino-1'-naphthalene
sulfonylamino)-propyl)-ammonium chloride

Methyl iodide and ethyldiisopropylamine are
obtained from Aldrich.

To a solution of bis-(3-aminopropyl)
methylamine (1 mmol) in dioxane/water (3 ml, 2/1) is
added 1 N NaOH (1 ml). With stirring, at 0°C, is added
di-tert-butyl dicarbonate (1.1 mmol) and the mixture is
stirred at 25°C for 30 minutes. The basic solution is
thoroughly extracted with chloroform and the combined
organic layers are dried over sodium sulfate and
evaporated. The product is dried by evaporation from
anhydrous dimethylformamide (5 ml). The residue is
dissolved in DMF and the solution treated with methyl
iodide (12 mmol) at 25°C for 1 hour. The solvent is
evaporated and the residue is dissolved in trifluoro-
acetic acid (5 ml). After 1 hour, the solvent is

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evaporated and the residue dissolved in pyridine (5
ml). To this solution is added ethyldiisopropylamine (2
mmol per mmol of primary, secondary, and tertiary amine
function) and the dansyl group is introduced by

5   treatment of the solution with 5-dimethylamino-
naphthalene-sulfonyl chloride (0.9 mmol) (obtained as in
Example XX) at 0°C, then allowing the mixture to stand
overnight at 4°C. The reaction product is purified as
in the previous paragraph by ion exchange

10   chromatography, and isolated as the hydrochloride salt.
          Alternatively, the dansylated reporter may be
treated directly with methyl iodide in dimethylformamide
and purified by ion exchange chromatography, as above.

15   Example 34
     General Method for the Introduction of the Fluorophore
          5-Dimethylamino-1-naphthalene-sulfonyl chloride
is obtained from Aldrich.
          To a solution of a starting polyamine (1 mmol)

20   in pyridine (5 ml) at 0°C is added 5-dimethylamino-1-
naphthalene-sulfonyl chloride (0.85 mmol) and the
mixture is stirred overnight at 4°C. The pyridine is
then removed at 25°C under reduced pressure and the
residue dissolved in 1 N HCl (5 ml) and applied to an

25   AG-50W ion exchange column. After washing with water
(10 ml), the product is eluted with a buffer comprised
of sodium acetate (0.1-2.0 M) and sodium chloride
(0.1-2.0 M) at pH 5. After collecting and concentrating
the fractions containing the product, the residue is

30   dissolved in water (10 ml) and applied to a Dowex 50-W
(Bio-Rad) ion exchange column and washed with water (10
ml), 0.5 N HCl (5 ml), and finally the product is eluted
with 6 N HCl. The hydrochloride salt of the product is

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obtained by removal of the solvent under reduced
pressure.

Example 35
Preparation of Diagnostic Reagent for
Detection of Herpes Simplex, Types I and II

The 16-base sequence at positions 462 to 477 of
the 2gD gene of Herpes simplex virus, types I and II,
5'-GCGGGGCTGCGTTCGG-3', comprises the selected target
sequence (Lasky & Downbenko, DNA (1984) 3:23). A
complementary reagent polymer composed of alternating
methylphosphonate/phosphodiester linkages is constructed
substantially according to the methods of Examples 23
ans 24.

The polymer is converted to the 5'-O-(6-amino-
hexyl)-oligonucleotide analog and coupled through the
aminohexyl spacer arm to an Affi-Gel 10 solid support
material, by conventional coupling methods.

The melting temperature of the polymer/analyte
duplex is determined using a synthetic oligonucleotide
having the 5'-GCGGGGCTGCGTTCGG-3' target sequence. This
target sequence, which is purchased, or constructed by
conventional methods, is mixed with a substantially
equimolar amount of the polymer (before polymer
attachment to the solid support) in annealing buffer (10
mM EDTA, 100 mM sodium phosphate, pH 7.2). This
solution is heated to 90°C and slowly cooled to room
temperature to effect annealing. The temperature is
then slowly raised and absorbance is recorded as a
function of the temperature. The melting temperature
(Tm) is taken as the temperature at which half the total
absorbance change has occurred.

Example 36

Detection of Herpes Simplex, Types I and II

An analyte sample, taken as a skin scrape of an
infected area, is suspended in 0.1 ml of EDTA-detergent
5   solution (10 mM EDTA, 1% w/v sodium dodecyl sulfate, pH
to 7.0), homogenized briefly in a micro tissue grinder
(Kontes #K-885470) and the homogenate filtered centri-
fugally in a microfilter (VWR Scientific #28151-807).
One-half ml of 4.5 M sodium trichloroacetate is added,
10   followed in a few seconds by 0.6 ml of ethanol. This
preparation is placed on ice for 30 min and then
centrifuged for 5 min at 10,000 g. The supernatant is
carefully decanted and discarded and the tube is gently
rinsed with aqueous 80% ethanol. The pelleted material
15   (often not visible) is resuspended in 0.05 ml of
annealing buffer (10 mM EDTA, 100 mM sodium phosphate,
pH 7.2) and added to an appropriate amount of diagnositc
reagent from Example XXI (estimated reagent polymer/
analyted molar ratio > 100). Annealing is carried out
20   at a temperature 8°C below the Tm of the polymer/target
duplex (predetermined as described in Example 35) for 30
min and then the diagnostic reagent is washed three
times with 2 ml volumes of annealing buffer. This
washing is conveniently carried out centrifugally in a
25   microfilter. After washing, the diagnostic reagent is
suspended in 0.2 ml of reporter solution containing 1 mg
of the fluorescent-diquaternary ammonium reporter, whose
synthesis is described in Example 34. The reporter
solution contains only NaCl at a suitable concentration,
30   predetermined as described above. The diagnostic
reagent is next washed three times with 2 ml volumes of
reporter-free binding solution. Finally, reporter is
eluted from the diagnostic reagent with 0.1 ml of 2 M
NaCl and the elutant assessed for fluorescence in a

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spectrofluorometer. The fluorescence from a control
sample lacking analyte is subtracted to provide a
quantitative measure proportional to the analyte present
in the initial sample.

5

Example 37
Preparation of Diagnostic Reagent
for Detection of the AIDS Virus

The 16-base sequence at positions 8441-8456 of
10 the ORF-2 gene of AIDS-associated retrovirus (ARV-2),
5'-GATGGGGTGGGAGCAG-3', comprises the selected target
sequence (Sanchez-Pescador, et al, _Science_ (1985)
_227_:484). A complementary polymer having carbamate-
linked subunits is constructed substantially according
15 to methods detailed in Example 21. Briefly, the
2'deoxyribonucleosides dA, dC, and dG are protected as
in Example X. These protected nucleosides plus
thymidine are then converted to their 5'amino
derivatives. Polymeric support is then prepared as in
20 Example 25 or 26, and subunits are linked sequentially
to this support by the method described in those
examples, to give a support-bound protected polymer
having the sequence: Support---CTGCTCCCACCCCATC-3'. In
the final step the base-protective groups are removed to
25 give the desired carbamate-linked diagnostic reagent.

The melting temperature of the polymer/analyte
is determined as follows. An RNA transcript is prepared
from single-stranded polynucleotide containing a
sequence complementary to the target sequence. This
30 target-containing RNA is suspended in annealing buffer
(10 mM EDTA, 100 mM sodium phosphate, pH 7.0) and added
to the above diagnostic reagent. The mixture is warmed
to 90°C and slowly cooled to room temperature to effect
annealing. Subsequently, the diagnostic reagent is

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placed in a small water-jacketed chromatography column
through which annealing buffer is slowly pumped. The
temperature is slowly raised while monitoring the
effluent for released RNA. The release temperature (Tr)
5   is the temperature at which the RNA is eluted from the
column. This Tr value often differs by one to a few
degrees C from the corresponding Tm determined by the
hyperchromic shift method described in Example XXI.

10   Example 38

Preparation of Enzymatic Reporter for Use

in Systems Employing Uncharged Diagnostic Reagents

A polycationic tail having a primary amine at
one terminus is prepared as described in Examples
15   XIV-XVIII, giving the structure:

$$H_2N(CH_2)_4 \qquad \underset{\underset{3}{CH_3}}{N(CH_2)_4} \qquad N(CH_3)_2$$
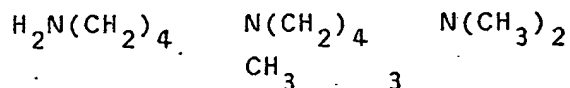
20   One mmole of this product is reacted for 24 hr at room
temperature in the dark with an excess of 4-fluoro-3-

In low light conditions the solvent is removed
under reduced pressure and the solid is triturated with
hexane to remove unreacted phenylazide. The solid is
25   next suspended in 10 ml of 0.1 M NaCl and cacodylic acid
is added to lower the pH to 7.2. Next, 5 mg of alkaline
phosphatase (Sigma Chem Co., #P5778) is added to 1 ml of
the foregoing tail solution and illuminated for 1 hr
with a high flux of 366 nm light. The resulting
30   reporter is dialyzed for 18 hr against a large volume of
buffer (0.1 M NaCl, 0.05 M sodium cacodylate, pH 7.0) to
remove cationic tail not linked to the enzyme. Bovine
serum albumin (1% w/v) and sodium azide (0.02% w/v) are
added to stabilize the enzymatic moiety of this

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tetracationic reporter. Storage of this reporter
solution is at 4°C in the dark.

Example 39
Detection of AIDS Virus

5   Five ml of blood suspected of containing the
AIDS virus is centrifuged and 2 ml of the cell-free
serum is added to 8 ml of 4.5 M sodium trichloroacetate
containing 0.1 mg polyadenylic acid (Sigma Chem Co
#P9403). After a few seconds 10 ml of ethanol is added
10 and the preparation chilled for 30 min in an ice bath
and then centrifuged for 5 min at 10,000 g. The
supernatant is carefully decanted and discarded. The
pelleted nucleic acid (often not visable) is washed with
aqueous 80% ethanol, drained well, and resuspended in
15 0.1 ml of annealing buffer (10 mM EDTA, 100 mM sodium
phosphate, pH 7.2) and added to an appropriate amount of
diagnostic reagent from Example 37 (estimated polymer/
target molar ratio > 100). Annealing is carried out at
20 a temperature 7°C below the Tr of the polymer/target
duplex (predetermined as in Example 37) for one hr and
then the diagnostic reagent is washed three times with 2
ml volumes of annealing buffer. This washing is
conveniently carried out centrifugally in a microfilter.
25   After washing, the diagnostic reagent is
suspended in 0.2 ml of an enzymatic-tetracationic
reporter solution prepared as in Example 38. After 30
sec the diagnostic reagent is washed three times with 2
ml volumes of reporter-free binding solution.
30 Thereafter, the diagnostic reagent is suspended in
developing solution (15 mM p-nitrophenyl phosphate, 0.5
mM MgCl, 1.0 M diethanolamine, pH 9.8) and incubated for
3 hr at 37°C. Reporter-generated p-nitrophenol is
quantitated spectrophotometrically. Corresponding

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absorbance from a control sample lacking analyte is
subtracted to provide a quantitative measure
proportional to the analyte present in the initial
sample.

5

    While the invention has been described with
reference to particular embodiments, it will be
appreciated that various changes and modifications can
be made without departing from the invention. For
10 example, the diagnostic reagent may include multiple
species of support-bound polymers, each specie designed
to bind to a different selected target sequence in
polynucleotide strands from different analytes, or the
diagnostic reagent may include two species of
15 support-bound polymers, with each specie designed to
bind to a different complementary strand of a duplex
analyte. These multi-specie reagents offer the
potential of detecting more than one analyte in a
diagnostic test; or of doubling the sensitivity of
20 detection of a single duplex analyte.

25

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WHAT IS CLAIMED IS;

1. A diagnostic reagent for use in detection
of a single-stranded polynucleotide analyte containing a
5 selected target sequence of bases, said reagent
comprising

    a solid support, and

    attached to the solid support, a plurality of
polymer molecules, each composed of a sequence of base
10 complementary recognition moieties adapted to hydrogen
bond to corresponding, in-sequence bases in such target
sequence, under selected binding conditions, and an
unbranched, substantially stereoregular backbone (1)
supporting the recognition moieties at positions which
15 allow hydrogen bonding between the recognition moieties
and the corresponding in-sequence bases in the target
sequence, and (2) having a backbone charge density which
is substantially less than that of the analyte under
such selected conditions.

20

2. The diagnostic reagent of claim 1, wherein
the polymer molecules are non-homopolymeric and
substantially stereoregular, and have the form:

$$
\begin{array}{cccc}
R_1 & R_2 & R_3 & R_n \\
| & | & | & | \\
B \sim & B \sim & B \sim & \ldots B,
\end{array}
$$
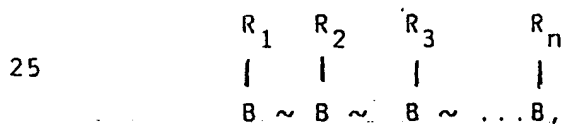
25

where:

    (a) where R_1-R_n are recognition moieties
selected from purine, purine-like, pyrimidine, and
30 pyrimidine like heterocycles effective to bind by
Watson/Crick pairing to corresponding, in-sequence bases
in the target sequence;

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    (b) n is such that the total number of
Watson/Crick hydrogen bonds formed between a polymer
molecule and target sequence is at least about 15;

    (c) the backbone moiety length ranges from 5 to
5 7 atoms if the backbone moieties have a cyclic
structure, and 4 to 6 atoms if the backbone moieties
have an acyclic structure; and

    (d) the backbone moieties support the
recognition moieties at positions which allow
10 Watson/Crick base pairing between the recognition
moieties and the corresponding, in-sequence bases of the
target sequences.

    3. The composition of claim 2, wherein the
15 recognition moieties are selected from the following
group:

20

25

30
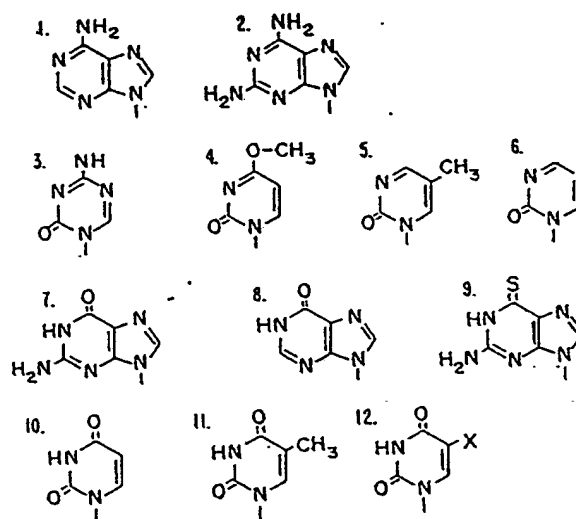
    where X=F, CL, Br, or I.

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5

10
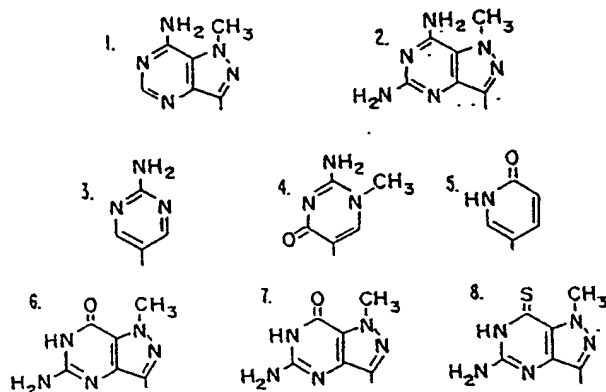
4. The composition of claim 2, wherein B is a
cyclic moiety and B ~ B are backbone moieties joined
predominantly by chemically stable, uncharged, achiral
linkages having one of the following forms:

15

20

25

30

where Y = O,S and E = $\overset{O}{\overset{\|}{C}}$ or $\overset{O}{\underset{\overset{\|}{\underset{O}{S}}}{}}$
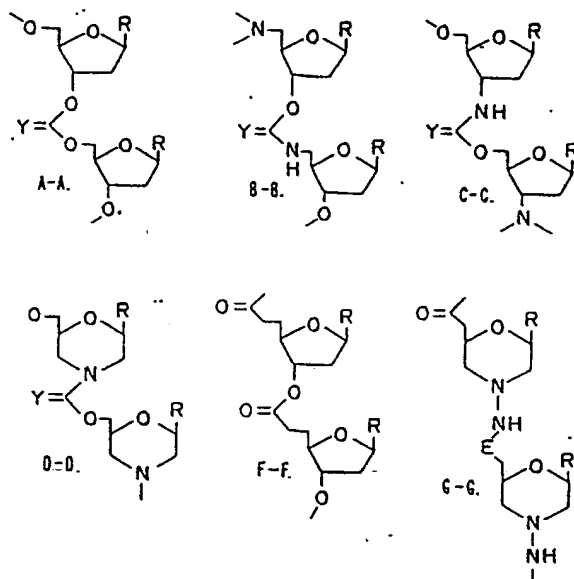
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5. The composition of claim 2, wherein B is an
acyclic moiety and B ~ B are backbone moieties joined
predominantly by chemically stable, uncharged, achiral
linkages having one of the following forms:

5

10

15
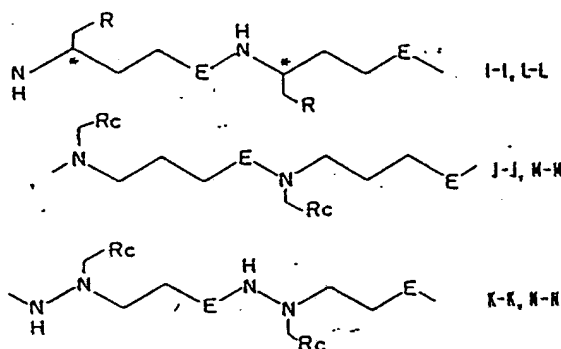
20   6. The reagent of claim 2, wherein the backbone
is composed of a series of subunit backbone moieties
joined by a sequence of negatively charged achiral
linkages alternating with one or more uncharged
stereoisomerically defined chiral linkages.

25

          7. The reagent of claim 6, wherein the
backbone moieties are joined by a sequence of
phosphodiester bonds alternating with one or more
stereoisomerically defined methylphosphonate linkages.

30

          8. A diagnostic system for determination of a
single-stranded polynucleotide analyte containing a
selected target sequence of bases, said system comprising

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a diagnostic reagent composed of a solid
support and, attached to the support, multiple polymers,
each composed of a sequence of base-complementary
recognition moieties adapted to hydrogen bond to
5   corresponding, in-sequence bases in the target sequence,
under selected binding conditions, and an unbranched,
substantially stereoregular backbone (1) supporting the
recognition moieties at positions which allow hydrogen
bonding between the moieties and the corresponding
10   in-sequence bases in the selected target sequence, and
(2) having a backbone charge density which is
substantially less than that of the analyte, under such
selected conditions, and
          molecules of a reporter which is composed of an
15   oligocationic tail adapted to bind electrostatically to
the charged backbone of the polynucleotide analyte under
preselected conditions in which the tail does not bind
to the less densely charged or uncharged backbone of
said polymers, and attached to the tail, one or more
20   reporter groups adapted to produce a signal by which the
presence of the reporter can be detected.

        9.   The system of claim 8, wherein the tail in
the reporter contains positive groups at intervals whose
25   spacing corresponds approximately to the spacing between
adjacent phosphate groups in the backbone of the
polynucleotide analyte.

        10.   The system of claim 9, wherein the tail
30   contains an oligocationic portion having the formula:

$$---N^+(R_1)(R_2)-(CH_2)_n-_m--N^+(R_3)(R_4)(R_5)$$
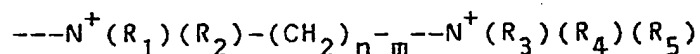
-126.-

where n = 2-5, m > O, and each $R_1 - R_5$ = H or alkyl
group.

11.   The system of claim 10, wherein the reporter
group in the reporter includes one or more fluorescent,
chromophore, enzyme, or radioisotope groups.

12.   A method for determination of a
polynucleotide analyte containing a selected target base
sequence, said method comprising
    providing a diagnostic reagent composed of a
solid support and, attached to the support, a plurality of
polymer molecules, each composed of a sequence of
base-complementary recognition moieties adapted to
hydrogen bond to corresponding, in-sequence bases in the
target sequence, under selected binding conditions and an
unbranched, substantially stereoregular backbone (1)
supporting the recognition moieties at positions which
allow hydrogen bonding between the moieties and the
corresponding in-sequence bases in the target sequence,
and (2) having a backbone charge density which is
substantially less than that of the analyte under such
selected conditions,
    mixing the reagent and an analyte-containing
sample under such selected conditions, to produce
sequence-specific binding of the analyte to the reagent
polymers,
    providing a reporter composed of an oligocationic
tail adapted to bind to the charged backbone of the
polynucleotide analyte, under preselected conditions at
which the tail does not bind to the less densely charged
or uncharged backbone of such polymers, and attached to
the oligocationic tail, one or more reporter groups

adapted to produce a signal by which the presence of the
reporter can be determined,

adding the reporter to the reagent under such
preselected conditions,

5   separating the reagent from unbound material
before or after adding the reporter, or both, and

determining the reporter signal of
analyte-associated reporter.

10   13. The method of claim 12, for use in
determination of a duplex nucleic acid analyte, wherein
said mixing is performed at a selected ionic strength of
less than about 0.2 M monovalent cation, in the presence
of a divalent cation chelating agent and at a temperature
15   above the melting temperature of such duplex analyte.

14. The method of claim 12, which further
includes establishing such preselected condition as the
highest salt concentration at which adequate binding of
20   reporter to the reagent-bound polynucleotide, at a given
temperature, occurs.

15. A reporter for use in detecting a
polynucleotide analyte, under preselected binding
25   conditions, comprising

an oligocationic tail containing two or more
cationic groups which are spaced for binding to negative
charges on the sugar-phosphate backbone of the
polynucleotide, under such binding conditions, and

30   one or more reporter groups attached to the tail
for producing a signal by which the presence of the
reporter can be detected.

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16.   The reporter of claim 15, wherein the
cationic groups in the reporter tail are spaced for
binding to consecutive negative charges in such
sugar-phosphate backbone.

17.   The reagent of claim 16, wherein the tail
contains an oligocationic portion having the general
formula:

$$---N^+(R_1)(R_2)-(CH_2)_n-_m--N^+(R_3)(R_4)(R_5)$$
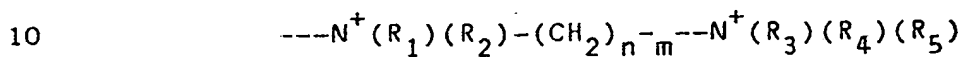
where $n = 2-5$, $m > 0$, and each R_1-R_5 = H or an alkyl
group.

18.   The reporter of claim 15, wherein the
reported group includes one or more fluorescent, enzyme,
radioactive, chromophoric, or radioisotope group.
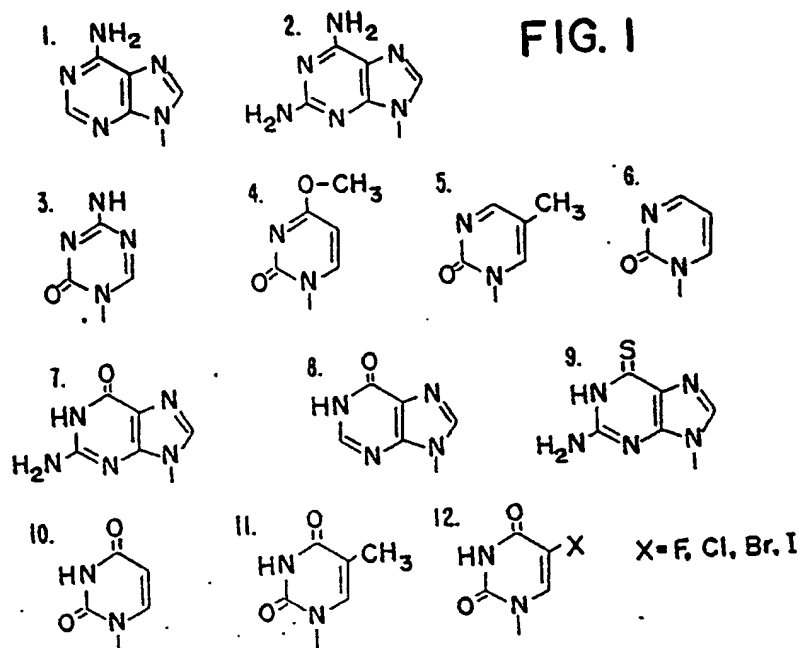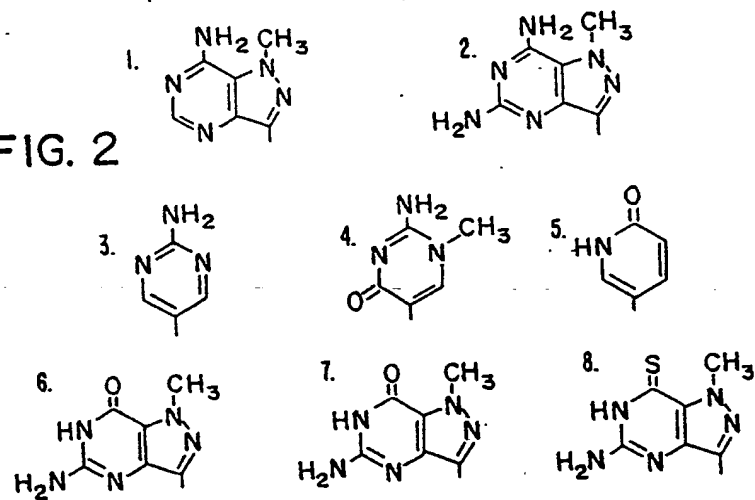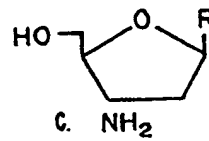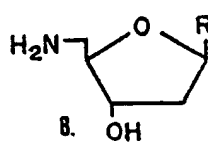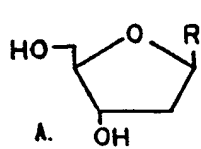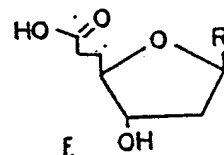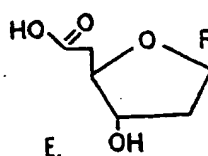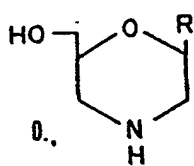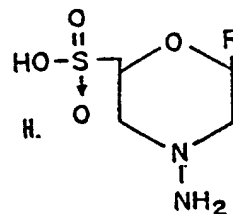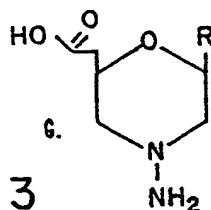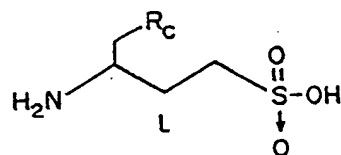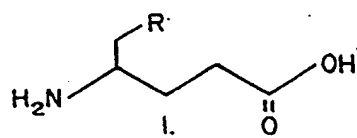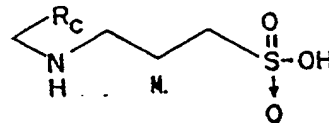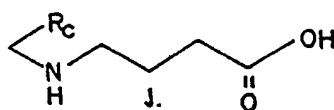
1/10

FIG. 1

FIG. 2

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FIG. 3
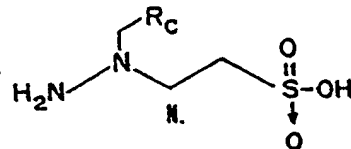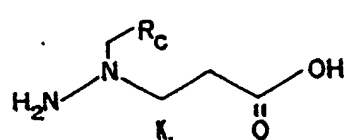
FIG. 4
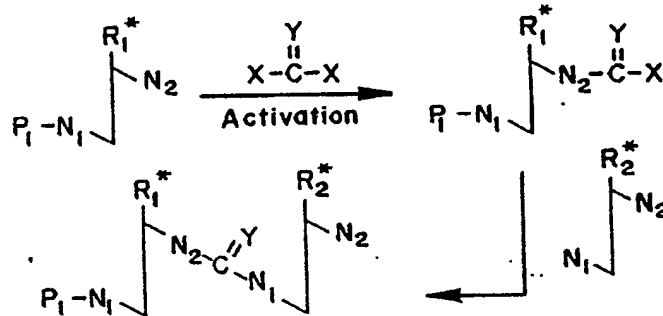
FIG. 5A
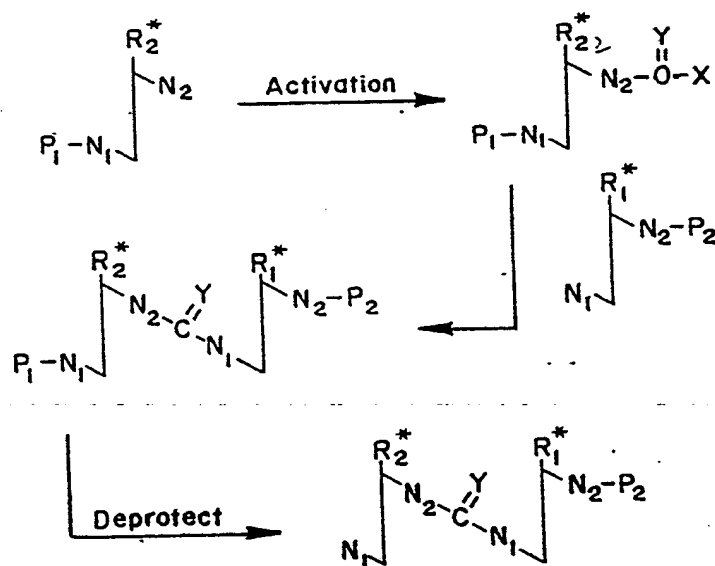
FIG. 5B
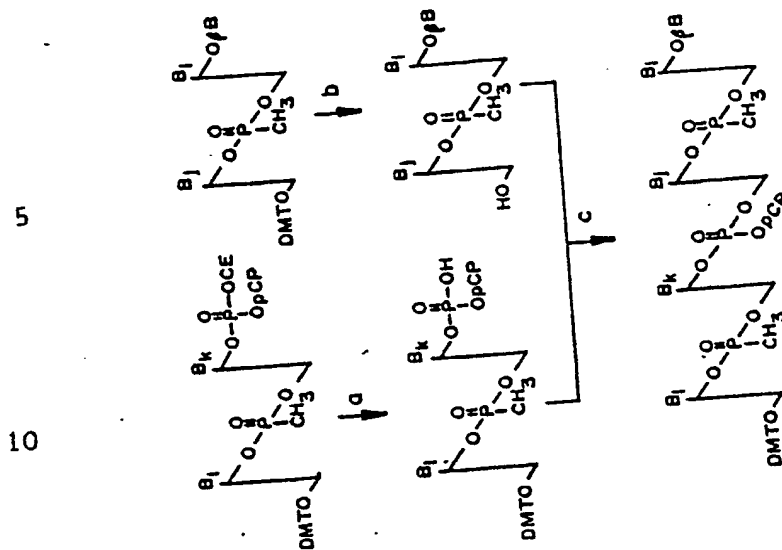
Figure 9

DMT. B_i, B_j, B_k, B_l, CE, pCP, βB defined as in Figure 3 and 4.

a = triethylamine/pyridine/water
b = benzenesulfonic acid/methanol/chloroform
c = mesitylenesulfonyl tetrazolide

FIG. 8

FIG. 6

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$$P_1 - N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E \xrightarrow[\text{Activation}]{X} P_1 - N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E-X$$

$$N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E$$

$$P_1 - N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E-N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E \longleftarrow$$

FIG. 7A

10

15

$$P_1 - N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E \xrightarrow[\text{Activation}]{X} P_1 - N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E-X$$

$$N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E-P_2$$

$$P_1 - N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E-N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E-P_2 \longleftarrow$$

$$\xrightarrow{\text{Deprotect}} N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E-N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E-P_2$$

20   **FIG. 7B**

25

$$P_1 - N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E$$

$$N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E-P_2$$

$$\xrightarrow{\text{DCC}} P_1 - N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E-N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E-P_2$$

$$\xrightarrow{\text{Deprotect}} N_1 \underset{\overset{|}{R_2^*}}{\overset{|}{}} E-N_1 \underset{\overset{|}{R_1^*}}{\overset{|}{}} E-P_2$$

30   **FIG. 7C**

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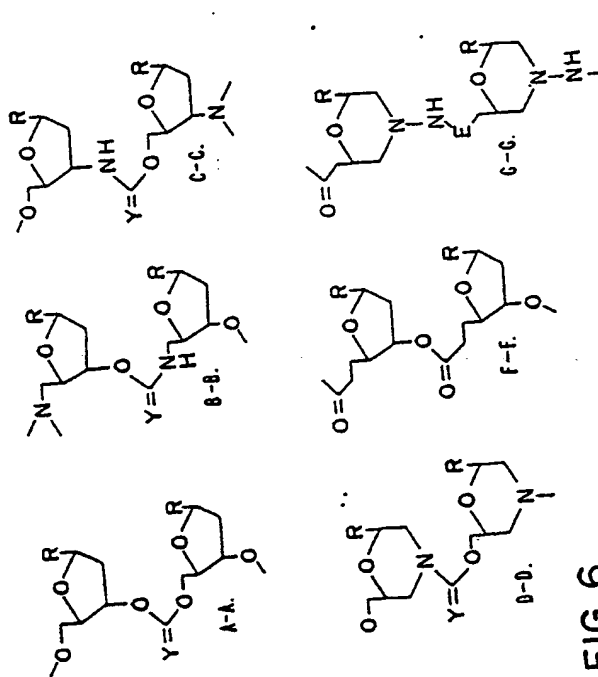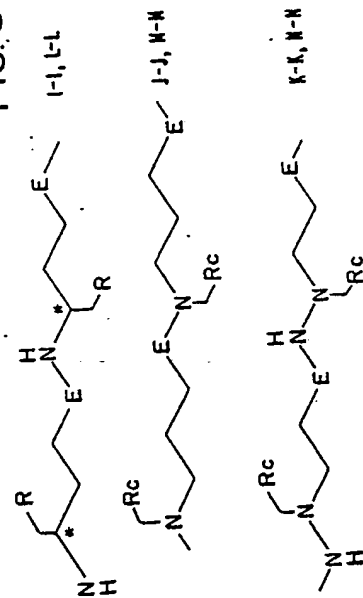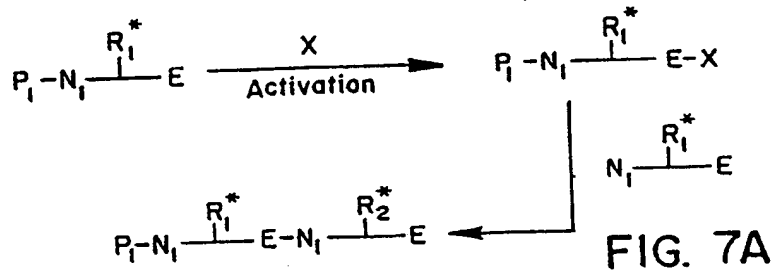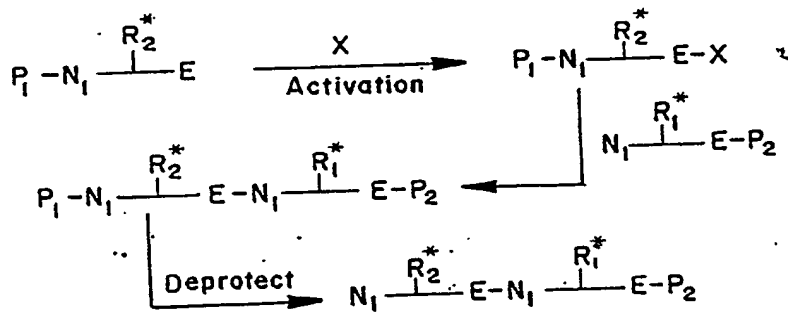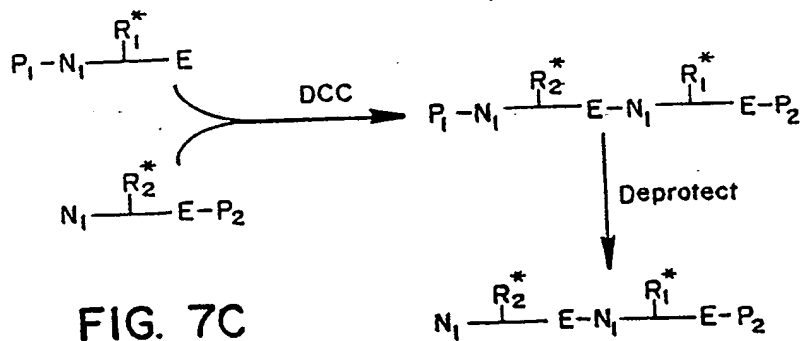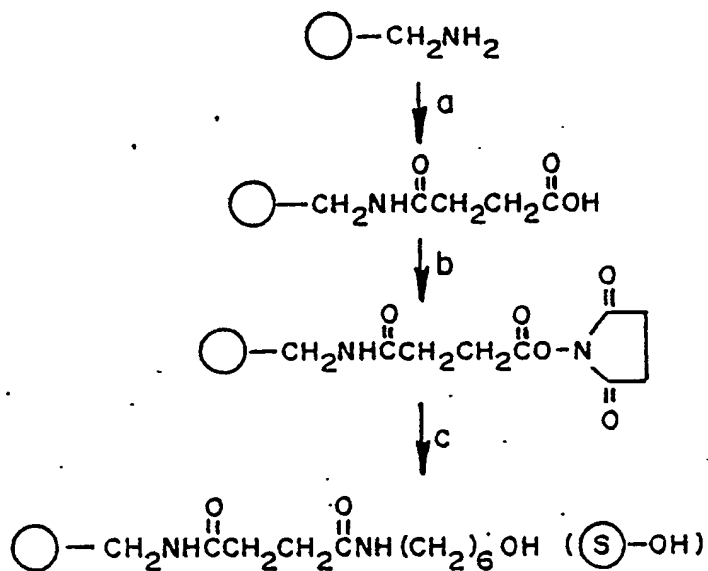
◯-CH₂NH₂ = aminomethylpolystyrene.
a = succinic anhydride
b = di(succinimido)carbonate
c = 6-aminohexanol

Figure 10

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$$HN(CH_2)_nCO_2H \xrightarrow{a} BOC\underset{CH_3}{N}(CH_2)_nCO_2H$$

BOC = tert-butoxycarbonyl

a = di-tert-butyl carbonate

b = carbonyl diimidazole

c = dimethylamine

d = trifluoroacetic acid

e = borane-tetrahydrofuran
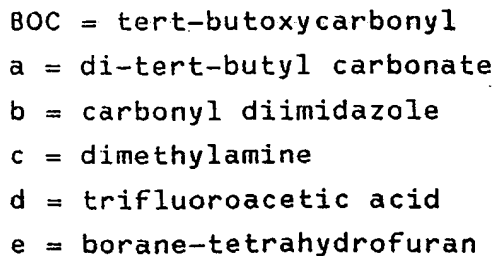
Figure 11

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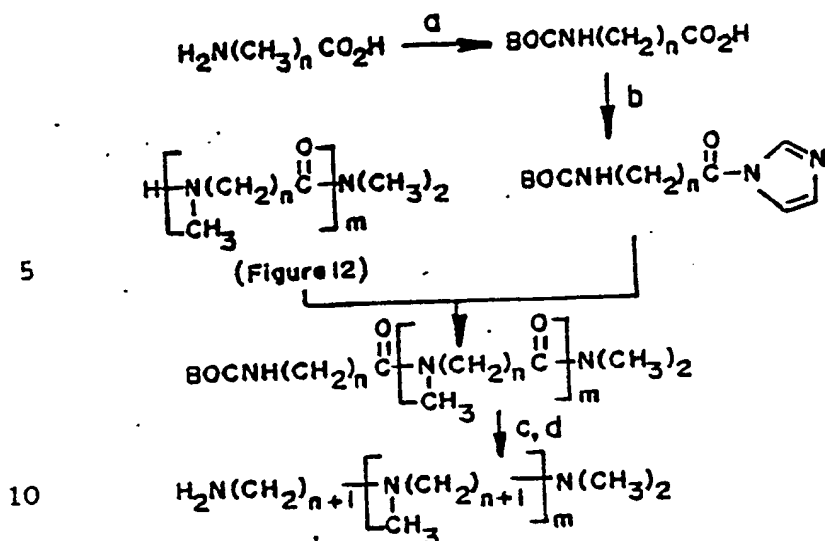
a = di-tert-butyl dicarbonate

b = carbonyl diimidazole

c = trifluoroacetic acid

d = borane-tetrahydrofuran

Figure 12
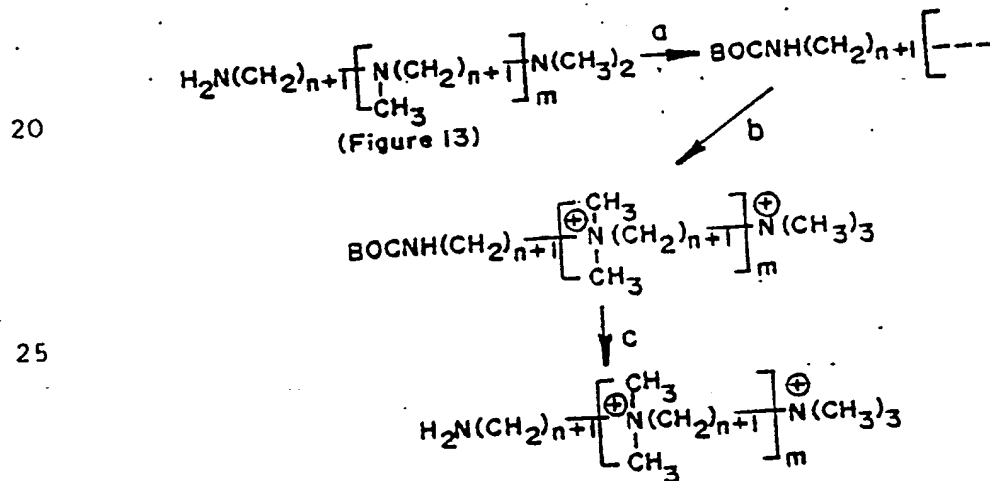
a = di-tert-butyl dicarbonate

b = methyl iodide

c = trifluoroacetic acid

Figure 13

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(Figure 14)

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light ↓ E(enzyme)

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Figure 14

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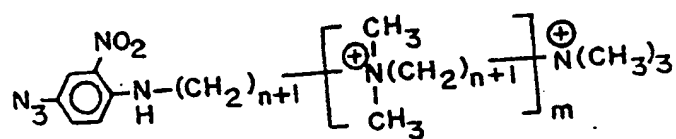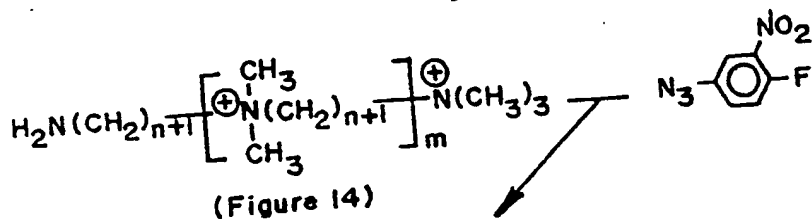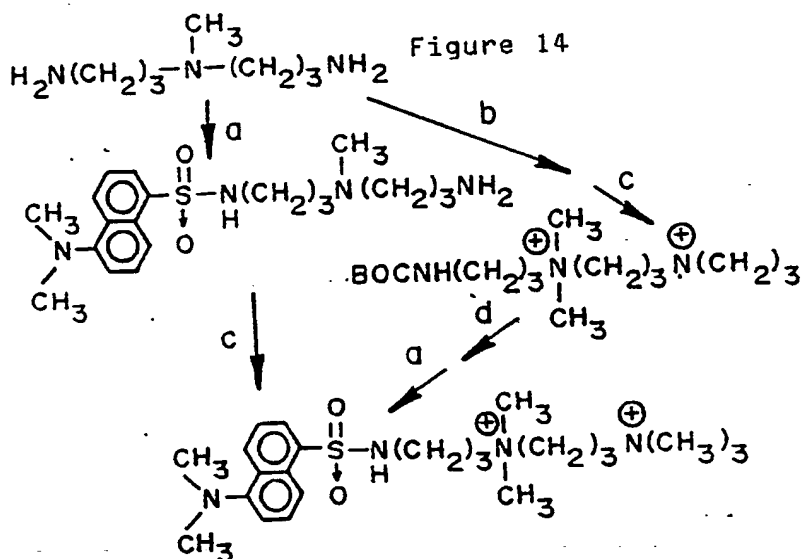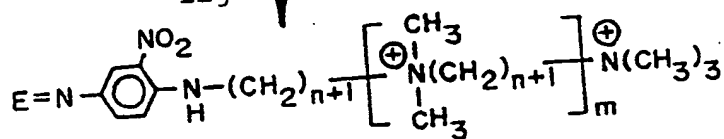
a = dansyl chloride

b = di-tert-butyl-dicarbonate

c = methyl iodide

30　d = trifluoroacetic acid

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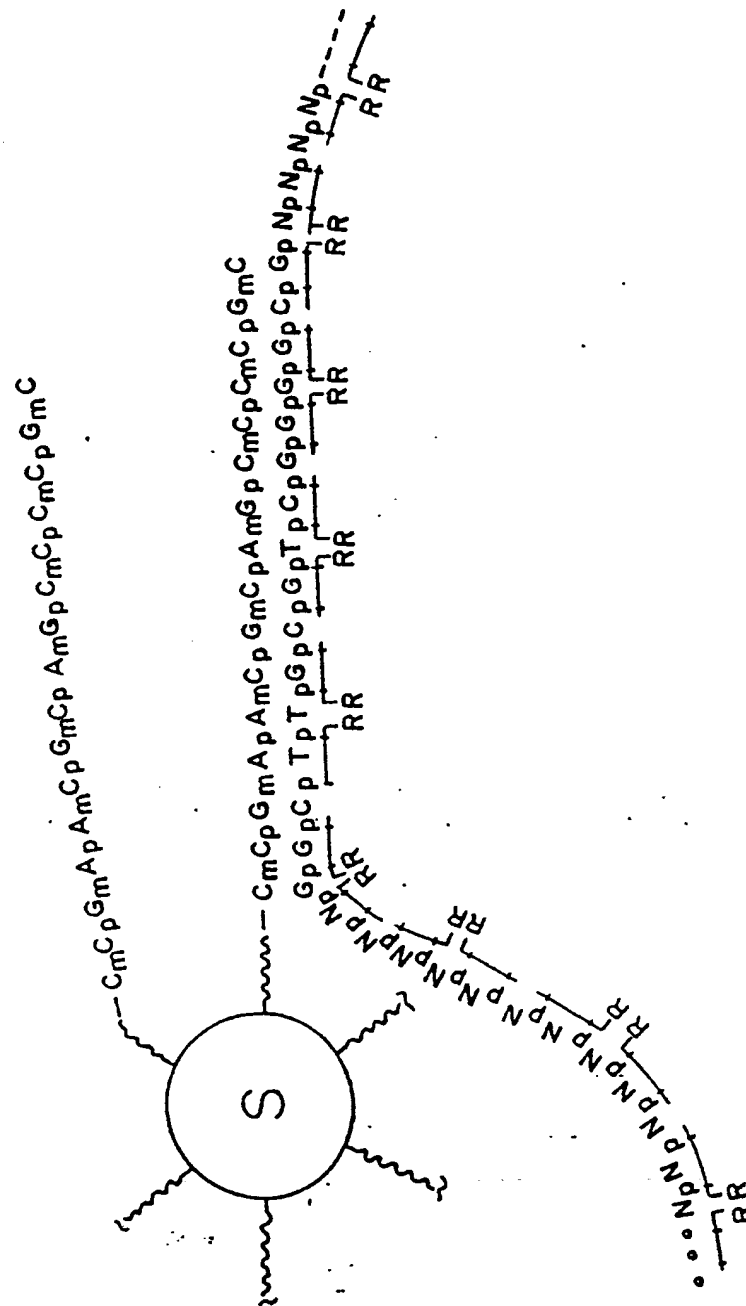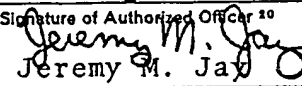
Figure 15

Figure 16

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

INT. CL.⁴   :   C12Q 1/68; C12P 19/34; C12N 15/00
U.S. CL.   :   435/6,91; 935/78

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴	
Classification System	Classification Symbols
U.S.	435/6,68,91,188,810 435/94,501,518,808 935/3,6,78

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁶

Computer Search: Chemical Abstracts 1967-1986;
Biosis 1977-1986; Derwent (WPI,WPIL,Biotechnology)

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
P,Y	Biochemistry, Volume 24, No. 22 issued August, 1985, A. Murakami et al, "Characterization of Sequence-Specific Oligo-deoxyribonucleoside Methyl-phosphonates and Their Interaction with Rabbit Globin mRNA," see pages 4045 and 4046.	1-12
Y	US,A, 4,395,486 (Wilson) 26 July, 1983, see column 3, lines 50-61.	13,14
Y	Nucleic Acids Research, Volume 12, No. 8, issued April 25, 1984, M. Renz et al, "A colorimetric method for DNA hybridization", see page 3435.	8-12,15-18

* Special categories of cited documents: ¹⁵

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ³	Date of Mailing of this International Search Report ⁹
05 June 1986	19 JUN 1986
International Searching Authority ¹	Signature of Authorized Officer ¹⁰
ISA/US	Jeremy M. Jay

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document,[16] with indication, where appropriate, of the relevant passages[17]	Relevant to Claim No[18]
X Y	Biochemistry, Volume 20, No. 7, issued March 31, 1981, P.S. Miller et al, "Biochemical and Biological Effects of Nonionic Nucleic Acid Methylphosphonates", see pages 1878 and 1879.	1 2-12
X Y	US,A, 4,507,433 (Miller) 26 March 1985 see column 1, lines 32-37.	1 2-12
X Y	Journal of Biological Chemistry, Volume 255, No. 20, issued October 25, 1980, P.S. Miller et al, "Oligothymidylate Analogues Having Stereoregular, Alternating Methylphosphonate/Phosphodiester Backbones", see page 9659.	1 2-12
Y	US, A, 4,362,531 (de Steenwinkel) 07 December 1982, see column 3, lines 50-64.	13,14
Y	US, A, 4,430,496 (Abbott) 07 February 1984, see column 6, lines 37-40.	8-12,15-18